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Nutritional Regulation of Protein Metabolism in the Small Intestinal Mucosa

by

Olasunkanmi John A. Adegoke

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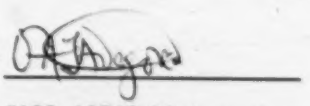

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'...Was not God's Laws,
His Gospel-Laws, in older times held forth
By types, Shadows and Metaphors? Yet loth
Will any sober man be to find fault
With them, lest he be found for to assault
The highest Wisdom: No, he rather stoops,
And seeks to find out what by pins and loops,
By Calves; and Sheep; by Heifers, and by Rams,
By Birds, and Herbs, and by the blood of Lambs,
God speaketh to him: And happy is he
That finds the light, and grace that in them be.'

John Bunyan 'Pilgrim's Progress'

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Nutritional Regulation of Protein Metabolism in the Small Intestinal Mucosa** submitted by **Olasunkanmi John A. Adegoke** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Nutrition and Metabolism**.

Dr. Vickie E. Baracos, Supervisor

Dr Michael I. McBurney

Dr Catherine J. Field

Dr Ronald O. Ball

Dr Chris I. Cheeseman

Dr Thomas C. Vary, External Examiner

Sept 11, 1998

Date

Abstract

The small intestine is an important component of whole body protein metabolism. It has one of the highest rates of protein turnover in the body, accounting for 9-15% of daily whole-body protein synthesis. Since the protein mass of the small intestine is sensitive to enteral (vs. intravenous) provision of nutrients, it was hypothesized that intestinal protein turnover would react separately to apical and basolateral stimuli. Therefore the objective of this research was to examine the roles of nutrients, applied apically and / or basolaterally, in regulating intestinal protein metabolism. A multi-segment luminal perfusion system for studying acute effects of luminal nutrients on intestinal mucosal protein metabolism was described and validated. Using this system, the effects of luminal nutrients (amino acid mixture, glutamine, glucose, short-chain fatty acids, β-hydroxy butyrate) on intestinal protein metabolism were examined in fasted piglets either under basal conditions, or when the overall nutritive status of the animals was modified by intravenous infusion of glucose (20mg / kg/ h). A luminal amino acid mixture or glutamine (30mM) rapidly (within 1 h) suppressed mucosal protein synthesis by 20-25% ($P< 0.05$); those substrates also significantly suppressed the expression of components of ATP-ubiquitin-proteasome proteolytic pathway by 30%. Luminal energy substrates (glucose, short-chain fatty acids and β-hydroxybutyrate) at concentrations of 20-50 mM had no effects on protein synthesis. Basolateral stimulus (glucose infusion) stimulated protein synthesis by 16% ($P < 0.05$) but had no effects on gene expression. The effects of luminal and basolateral treatments did not interact. It was concluded that: 1) Luminal amino acids may acutely regulate intestinal mucosal protein metabolism by energy-

conserving mechanisms involving suppression of both protein synthesis and degradation, 2) Positive mucosal protein balance after feeding may result from decreased protein catabolism and a systemically mediated rise in protein synthesis.

To my wife Theresa,

Who, true to her name, **Ngozi Chukwuka**, has been to me from the Lord a source of great blessings

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List of Abbreviations and Symbols

E1: Ubiquitin activating enzyme

E2: Ubiquitin conjugating enzyme

E3: Ubiquitin protein ligase

GLP: Glucagon-like peptide

IGF: Insulin-like growth actor

IL-1: Interleukin-1

Ks: Fractional rate of protein synthesis

mRNA: Messenger RNA

OKG: Ornithine α-ketoglutarate

PBS: Phosphate buffered saline

S_b: Protein-bound tracer specific radioactivity

SCFA: Short-chain fatty acids

SDS: Sodium dodecyl sulphate

S_f: Intracellular free tracer specific radioactivity

Sr: Specific radioactivity

TPN: Total parenteral nutrition

TNF: Tumor necrosis actor

tRNA: Transfer RNA

CHAPTER 1

Introduction

General Features

The small intestine consists of the mucosa layer which borders the intestinal lumen, and the underlying serosa layer. The layer between these two is the submucosa which consists mainly of connective tissues and blood vessels (Fig. 1-1). The mucosa can be divided into the epithelium, the laminal propria, the muscularis mucosae while the serosa consists of an inner circular and the outer longitudinal muscle layers (Berne and Levy, 1988. The Intestinal epithelium is made up of mainly the absorptive epithelial cells. Other cells present in the epithelium include immune and endocrine cells, with each of these two cell populations constituting up to 15% and 1%, respectively (Elson and Beagley, 1994; Hermiston et al., 1994). The laminal propria contains diverse sets of cell populations including connective tissue and immune cells. (Berne and Levy. 1988). The muscularis mucosae is the thin innermost layer of the intestinal smooth muscle. Its contractions throw the mucosa into folds and ridges.

Protein Metabolism in the Small Intestine

The protein mass of an organ is a function of the rates of protein synthesis and protein degradation. If the rate of protein synthesis is greater than the rate of protein degradation, the organ has positive protein balance and growth can be observed. Protein balance will be negative if the reverse is the case. Thus as in all tissues, the growth of the small intestine is a function of the protein metabolism status of the organ.

The small intestine has one of the highest protein turnover rates in the body. In the young pig and preruminant lamb, although it is only about 3% of body weight, the small intestine contributes disproportionately 9-12% of the total protein synthesized per day (Attaix and Arnal, 1987; Simon et al., 1982). This tissue is also the site for the production of enzymes and nutrient transporters, proteins that are important in digestion and absorption. In addition, the small intestine produces a number of regulatory hormones and growth factors. These include glucagon-like peptides 1 and 2 (GLP-1 and –2), glucose-dependent insulinotrophic polypeptide, secretin, insulin-like growth factors, all of which are important, not only in locally regulating the growth of the small intestine (Drucker, 1997, Weir, 1995) but also in regulating whole body carbohydrate, protein and lipid metabolism (Shalev, et al., 1997; Holst, 1994; Knapper et al., 1995). Finally, this tissue also produces mucus glycoproteins (mucins) which line the surface of the intestinal lumen. This glycoprotein lining protects the epithelium against mechanical damage and chemical irritants (Tytgat et al., 1993) and serves as the first barrier against the influx of potential pathogens from the intestinal lumen into the systemic circulation (Lamont, 1992).

There are longitudinal differences in protein metabolism in the small intestine. In both ruminants and monogastrics, the rate of protein synthesis is higher in the proximal small intestine (duodenum and jejunum) compared to ileum (Eisemann et al., 1989; Marway et al., 1993a). Within each segment, the mucosa has a higher rate of synthesis compared to the serosa (Garlick et al., 1980; Marway et al., 1993a). Also, different proteins are synthesized in the two layers. The mucosa is the layer directly in contact with nutrients and is therefore the site for production of enzymes, transporter proteins and glycoproteins

(Alpers and Kinzie, 1973). The serosa serves mainly to give structural support to the intestine and is therefore relatively richer in contractile proteins (Marway and Preedy, 1994; Marway et al., 1993a). Within the mucosa there are also differences in the rate and kinds of protein synthesized and their functions. Although in healthy rats protein synthesis rates were identical in cells isolated from the tip to the base of the villous (Higashiguchi et al., 1993), higher rates of protein synthesis have been reported in crypt (compared to villous) cells isolated from septic rats (Higashiguchi et al., 1994). Cells in the middle to the tip of the villi are the ones directly in contact with nutrients. Therefore, disaccharidases, peptidases and other proteins involved in digestion and absorption are synthesized in the villi cells while proteins like thymidine kinase are located in the crypt cells (Alpers and Kinzie, 1973).

Some of the proteins synthesized by the intestinal mucosa are exported (Smith, 1984). This is of significance in the choice of methods used to estimate protein synthesis and degradation in the small intestine. In measuring protein synthesis, if the time of tracer administration is too long, some of the proteins synthesized might have been secreted and therefore, protein synthesis would be underestimated. The implication for protein degradation lies in the fact that the disappearance of proteins from intestinal tissues is not solely due to degradation, and unless this is accounted for, degradation will be overestimated.

Regulation of Small Intestinal Protein Metabolism

Regulation of Protein Synthesis

Protein synthesis in the small intestine is regulated by nutritional, physiological, pathological and endocrine factors. The different factors, listed in Table 1-1, are discussed below.

Nutritional Regulation

This is the most studied aspect of intestinal protein metabolism. Amongst the nutritional factors known to affect protein synthesis in the small intestine are fasting-refeeding and protein calorie-restrictions. Fasting decreased protein content of the intestine and, in young animals, suppresses the fractional rates (Ks) of protein synthesis by about 40% (Burrin et al., 1991a; McNurlan et al., 1979). It also reduced translational efficiency (amount of protein synthesized per unit of RNA per unit time) in whole intestine as well as in the mucosal and serosal layers. The suppressive effect of fasting on protein synthesis is reversible with re-feeding (Davis et al., 1996). This regulation however appears to be age-dependent, as in studies with old rats and adult humans the effects of fasting is either modest or absent (Burrin et al., 1991a; Bouteloup-Demange et al., 1998; Samuels et al., 1996). The effect of re-feeding is also more pronounced in the 1-week old than in the 3-week old piglet (Davis et al., 1996). The relatively refractory response of adult intestine to fasting is thought to be due to the fact that older animals have greater stores of energy (as fat) and can therefore withstand fasting with limited effects on protein synthesis.

McNurlan and Garlick (1981) and Wykes et al. (1996) have also shown that feeding a protein-restricted diet for 1-8 weeks resulted in a fall in intestinal mucosal and serosal Ks by 20-50% in young rat and pig. Losses were also seen in tissue protein, RNA and DNA, and the effects were more pronounced in the mucosa (McNurlan and Garlick 1981).

In a study with neonatal piglets, intestinal protein and RNA masses, fractional synthetic rate, translational efficiency were higher in milk fed than water-fed piglets, and higher still in colostrum- vs. milk-fed piglets (Burrin et al., 1992). The effects of colostrum were attributed to its content of non-nutrient growth factors like insulin and insulin-like growth factors (Burrin et al., 1995). In a study with 8-week old weaned lambs, Attaix et al. (1992) showed that compared to age-matched milk-fed lambs, fractional protein synthetic rates were higher in the small intestine of weaned lambs, the increase being due to increase in synthetic (translational) efficiency and ribosomal capacity (μg RNA / mg cell protein).

The effects of a number of specific nutrients on intestinal protein synthesis have also been studied. Glutamine is known to be involved in the regulation of different aspects of metabolism under different catabolic states. Its inclusion in TPN (Kaibara et al., 1994; Li et al., 1994) or oral diet (Fahr et al., 1994) has been shown to enhance immunoregulation of tumor growth, reduce whole body protein breakdown, and prevent TPN-induced increases in intestinal permeability. Glutamine is a selective oxidative fuel of the small intestine (Windmueller and Spaeth, 1978). Compared to glycine or alanine, glutamine, when given as 25-30% w / w of total parenteral nutrition (TPN) amino acids enhanced

weight, DNA content, villous height and protein synthesis of the small intestine (Grant and Synder, 1988; O' Dwyer et al., 1989; Stein et al., 1994). Burrin et al. (1991, 1994), however, could not find any effect of glutamine or glutamic acid, supplied as 5 to 40 % (w/w) of TPN total amino acids, on either protein or DNA content, nor on specific activities of lactase, sucrase or maltase in jejunum and ileum.

Weber et al. (1989) have shown that, in the fed rat, luminal perfusion of 10-cm jejunal segment for 1.75h with 56 mM glucose increased jejunal mucosal protein synthesis by 20 and 37% with intravenous and luminal delivery of tracer, respectively. The stimulatory effect of glucose was seen in both fed and fasted states with luminal isotope deliver but was abolished in fasted animals when tracer was administered intravenously.

In a study with burned rats, providing in TPN non-protein calorie as 50% triacetin and 50% long chain triglycerides or as 100% long chain triglyceride promoted N-balance, increased structural components of both small and large intestines, and decreased the development of intestinal mucosal atrophy associated with conventional TPN in burned injury (Karlstad et al., 1992). Other authors have examined the effects of structured lipid (consisting of 17, 18, 10, 5, 17, and 33 % capryllate, caprate, palmitate, stearate, oleate, and linoleate, respectively), long-chain triglycerides (consisting of 14, 6, 24, 51, and 4 % of palmitate, stearate, oleate, linoleate, and linolenate, respectively) and sodium butyrate on intestinal protein synthesis in intravenously-fed rats. Compared to glucose, inclusion of structured lipid, long-chain triglycerides or sodium butyrate as 50, 50, or 9% of non-protein energy increased jejunal mucosal protein synthesis about 2-fold (Stein et al.,

1994). The effects of structured lipid and butyrate were attributed to these substrates being precursors of ketone bodies which in turn are important fuels for the intestinal mucosa. In spite of this stimulation however, protein synthesis was higher in orally fed rats compared to any of the TPN treatments (Stein et al., 1994). Tappenden (1997) also showed that inclusion of short-chain fatty acids (acetate, propionate and butyrate) in TPN increased ileal, but not jejunal, RNA and DNA relative to a TPN solution lacking these nutrients. The effects of triglyceride composition on jejunal protein synthesis have also been shown to depend on both the route (oral or TPN) of administration and the metabolic state of the animal (Schwartz et al., (1994). Thus, in a hepatectomy model with TPN, 50% of triglycerides supplied as medium chain triglycerides was more effective in stimulating jejunal protein synthesis than any other medium chain / long-chain triglycerides ratio examined. There were no differences amongst the different ratios when the rats were fed orally. In another model, (femoral fracture) 40% medium-chain triglycerides was the most effective ratio only when the rats were fed orally (Schwartz et al., 1994).

Other nutrients shown to have regulatory effects on growth and protein metabolism of the small intestine are ornithine α-ketoglutarate (OKG) and nucleic acids. Enteral OKG increased intestinal Ks and protein deposition relative to a glycine diet (Le Bricon et al., 1995). Another study showed that an enteral diet supplemented with nucleotide-nucleoside mixture reduced bacterial translocation and endotoxin-related mucosal damage in mice consuming protein-free diets (Adjei and Yamamoto, 1995). Lopez-

Navarro et al. (1995) have also shown that feeding rats a nucleotide-free diet led to a fall in intestinal Ks and DNA concentration.

Studies referred to above generally involved long term feeding trials. As such, it is not clear whether the observations made were as a result of the direct effects of the nutrients, or indirectly due to the effects of gut and systemic hormones released in response to these nutrients. Using isolated enterocytes, Higashiguchi et al. (1993) showed that, compared to an unsupplemented medium, 0.67 mM glutamine stimulated enterocyte protein synthesis. Because of the limitations associated with incubation of enterocytes (Watford et al., 1979), the relevance of the observations to *in vivo* phenomena remains to be shown. Thus, the acute effects of nutrients on intestinal protein metabolism are unknown.

Endocrine Regulation

Only a few studies have examined the effects of hormonal factors on intestinal protein metabolism. However, some of the feeding trials referred to above indicate that hormones like insulin-like growth factors (IGFs) and insulin may be important in the regulation of intestinal protein metabolism. The fasting-refeeding study of Davis et al. (1996) for instance indicates that the increase in protein synthesis associated with re-feeding correlated with an elevated circulating insulin concentration. Studies with steers and neonatal piglets have shown that growth hormone increases mass and protein content of the small intestine without any affects on Ks (Wester et al. 1998, Eiseman et al., 1989). Mice expressing a bovine growth hormone fusion gene exhibit increased jejunum and duodenum weights with increased villus height but with no effect on crypt depth

(Drucker 1997). Thyroid hormones have also been implicated in increased intestinal protein content and synthesis (Marway et al., 1994; Simon et al., 1982). Whether these growth factors have direct effects on intestine is presently not clear.

GLP–2, insulin-like growth factors-1 and –2 (IGF-1 and –2) epidermal growth factor have been shown to promote intestinal cell proliferation and intestinal growth. IGF-1 and its receptors in particular are expressed in the small intestine and thus may exert their intestinotrophic effects through endocrine or paracrine mechanisms (Drucker 1997). A number of studies indicate that IGF-1 has anabolic effects on the small intestine (Steeb et al., 1994, Yang et al, 1994). Lo and Ney (1996) have demonstrated stimulatory effects of IGF-1 on jejunal mucosa and muscularis Ks in TPN-fed surgically stressed rats. Another study with orally fed healthy rats could not demonstrate any effects of IGF-1 on whole duodenal Ks (Steeb et al., 1994), indicating that the effects of IGF-1 may depend on the pathophysiological stage of the animal. In a work with jejunal crypt cells, IGF-1 and –2 stimulated protein synthesis when the incubation medium included dexamethasone, with IGF-1 being about 10 times as potent compared to insulin and IGF-2. (Park et al., 1990, 1992).

GLP-2 stimulates crypt cell proliferation with an increase in intestinal mass (Drucker et al., 1996). It promotes intestinal growth after subcutaneous, intraperitoneal, or intramuscular administration, and increases small bowel mucosal mass in a dose-dependent fashion (Tsai et al., 1997). Moreover, a recent study shows that parenteral nutrition-induced gut atrophy can be prevented when TPN is co-infused with GLP-2

(Chance et al., 1997). In another study, GLP-1 administration to healthy humans decreased whole body protein synthesis and degradation (Shalev, et al., 1997). The decreased protein synthesis was attributed to hypoaminoacidemia caused by GLP-1 induced hyperinsulinemia. However, GLP-2 is the more potent peptide in terms of gut growth and it is not insulinotrophic (Drucker, 1997). The effects of this peptide on intestinal and whole body protein metabolism remain to be studied.

Other hormones and growth factors which have been shown to promote intestinal epithelial cell division and mass include epidermal growth factor, keratinocyte growth factor and the cytokines interleukin-3, -11, and –15 (see Drucker 1997 for a review). However the effects of most of these growth factors on intestinal protein metabolism remain to be determined, except for the effect of interleukin-1 which is described in a section below.

Effects of Physiological Stage

Apart from the effects of components of feed given to weaned animals, like other organs and tissues in the body, stage of development is also know to affect the growth of gastrointestinal tissues (Davis et al., 1996, Burrin et al., 1991a). Between birth and weaning in rats, fractional protein synthesis in stomach, pancreas and intestine rose by 80-600% while absolute rates rose 26- to 95-fold. Such a regulation is presumably necessary to ensure the gastrointestinal tract is able to digest and absorb sufficient nutrients so that other organs and tissues in the body can achieve the potentials for growth that exist immediately after birth. Along this line, various studies (Baillie and

Garlick, 1991; Davis et al., 1996) have shown that protein synthetic rate in skeletal muscle is higher in younger animals, and that the response of muscle protein synthesis to re-feeding after fasting is much more pronounced in younger animals (Davis et al. 1996).

Studies with pregnant (Millican et al., 1987) and lactating animals (Baracos et al., 1991; Barber et al., 1990; Millican et al., 1987) indicate that total small intestinal protein synthesis and accretion increased compared to that observed in virgin or dry animals. A greater increase in intestinal protein deposition was found in lactating compared to pregnant mouse (Millican et al., 1987). This is presumably necessary so that the intestine is adapted to supply nutrients for the elevated demand of the body for nutrients during these physiological stages.

Effects of Pathological Stage and Cytokines

Unlike other major organs in the body, one of the features of intestinal protein metabolism is an increased rate of protein synthesis during pathological stages. In a study with streptozotocin-treated rats, diabetes was associated with increased intestinal length and protein, DNA, and RNA contents in the small intestine. It was also associated with an increase in the absolute rate of mucosal protein synthesis (McNurlan and Garlick, 1981). During sepsis (von Allmen et al., 1992; Higashiguchi et al., 1994; Breuille et al., 1994; Yoshida et al., 1992), or celiac diseases (Nakshabendi et al., 1996), protein synthesis in whole small intestine or intestinal mucosal and serosal layers generally rose by 15-180%, depending on the region of the small intestine, and whether the mucosal or serosal layer was studied. Another study, however, showed that five days after the placement of septic

focus in the abdominal segment, there were no effects on small intestinal mucosal or serosal Ks (Cooney et al., 1996).

In skeletal muscle and liver, cytokines and stress hormones have been implicated in alterations in protein metabolism during pathological states (Biolo et al., 1997; Cooney et al., 1997). Data on the regulation of intestinal protein metabolism by cytokines are scanty. However, detection of increased interleukin gene expression in mucosal samples from malnourished rats (Lyoumi et al., 1998), and from animals and patients with maladies like inflammatory bowel disease has raised the possibility that cytokines may be involved in the regulation of intestinal growth (Drucker, 1997). Cytokines have been implicated in sepsis-induced increase in intestinal protein synthesis. For example, sixteen hours after intravenous administration of interleukin-1, Ks in jejunal mucosal and serosal layers were increased by 40-90% (von Allmen et al., 1992). Similarly, tumor necrosis factor-α adminstration increased jejunal, but not ileal, mucosal Ks by 25%. The cytokines had no effects on seromuscular layer Ks. In another study, however, 5-day administration of IL-1 receptor antagonist (IL-1ra) to septic animals did not have any effects on mucosal or serosal Ks (Cooney et al., 1996). Control animals receiving the IL-1ra had higher rate of serosal protein synthesis when compared to non-treated control. Although questions still remain as to the extent of significance of cytokines in regulating intestinal metabolism, there appears to be evidence to indicate their significance in the regulation of growth and functions of the gastrointestinal tract. Further studies will be needed to demonstrate the importance of cytokines in regulating intestinal protein metabolism. Increased intestinal protein synthesis in pathological states is difficult to explain because

protein content of the tissue was unchanged or fell (von Allmen et al., 1992; Samuels and Baracos, 1992). Moreover activities of enzymes like maltase and lactase fell (Ameh, 1991), and the transport of many amino acids, including leucine, glutamine, glutamic acid, proline, arginine and alanine fell during sepsis and endotoxaemia (Gardiner and Babul, 1993; Salloum and Copeland, 1991). At least for glutamine, the fall in amino acid transport was due to reduced Vm, possibly indicating a reduced amount of transporter proteins (Salloum and Copeland, 1991).

Increased protein synthesis in the intestine in pathological states without a corresponding increase in tissue mass or function may be due to an associated increase in protein degradation, or increase in the synthesis of export protein, or an increase in the rate of cell turn-over. No published works were found on the effects of pathological states on intestinal protein degradation. However, many pathological states are associated with increase in intestinal cell proliferation (Rafferty, et al., 1994) and increase in production of secreted proteins such as peptide YY (Higashiguchi et al., 1994), a peptide that has been reported to promote intestinal cell proliferation (Drucker 1997).

Studies on the effects of surgical stress and anaesthetic agents on intestinal protein metabolism have shown that surgery did not affect whole jejunal (Bakic et al., 1988; Preedy et al., 1988) or suppressed by 30% mixed jejunal seromuscular and seromuscular sarcoplasmic, myofibrillar and stromal protein synthesis (Marway and Preedy, 1994). Thus effects of surgery on intestinal protein synthesis appear to be limited to the structural component of the tissue with no effect on mucosa.

Effects of Alcohol

A number of studies has shown that alcohol, as 36% of calorie or at 8% inclusion in incubation medium, reduces protein contents, especially contractile proteins, and suppressed protein synthesis in both intestinal epithelial cells and jejunal serosal layer (Marway et al., 1993a; Preedy and Peters, 1990; Lau et al., 1990). Such a suppression is thought to be involved in the alteration of intestinal motility caused by alcohol consumption (Preedy and Peters, 1990).

Cellular Hydration State

Changes in cellular hydration state, resulting from exposure to either anisosmotic environments, nutrients or hormones, is one of the mechanisms involved in regulation of cellular metabolism (Busch et al., 1996; Häussinger, 1996). In hepatocytes and mammary gland explants, cell swelling increased protein synthesis and suppressed proteolysis while cell shrinking has opposite effects (Busch et al., 1996, Millar et al., 1997). Furthermore, the effects of hormones and amino acids on hepatic protein synthesis and proteolysis can be mimicked quantitatively when the cell volume changes observed in response to these effectors are induced to the same degree by anisosmotic exposure (Häussinger, 1996). Since epithelia cells of intestinal mucosa are exposed to variations in composition and tonicity of the fluid bathing them (Bedford and Leader, 1993) such variations will be expected to regulate intestinal mucosal metabolism. A study with isolated rat colonocytes showed that isoosmolar (NaCl) solution suppressed protein synthesis to the same degree as 30mM short-chain fatty acids (SFCA (Marsman and McBurney, 1996). Further studies

are needed on the significance of cellular hydration state in regulating intestinal metabolism.

Effects of Route of Supply of Nutrients

Because intestinal mucosal cells can take nutrients from both the intestinal lumen and the blood for incorporation into proteins (Fig. 1-2), the route of nutrient delivery is also important in regulating intestinal growth. Several studies have shown that intravenous nutrition results in small intestinal atrophy, with concurrent decreases in protein and DNA content, villus height and surface area. These effects may lead to a decrease in digestive capacity (Burrin et al., 1994) and weakening of the intestinal barrier which could lead to an increase in the incidence of bacterial translocation (Burke et al., 1989; Li et al., 1994). TPN-feeding is also associated with a decrease in intestinal mucosal protein synthesis when compared to oral nutrients (Stein et al., 1994, Dudley et al., 1998), although absolute synthesis rate of brush-border lactase phlorizin hydrolase was maintained in the intestine of young piglets (Dudley et al., 1998). Also associated with TPN-feeding is increased activities of cathepsins B, H, and L, enzymes involved in proteolysis. However the activities of these enzymes changed more in ileal mucosa whereas jejunum atrophied more (Kishibuchi et al., 1995), probably indicating the involvement of other proteolytic systems.

It is generally thought that the catabolic effects of TPN feeding are due to the absence of luminal nutrients. However, a recent study shows that co-infusion of GLP-2 with TPN removes all TPN-associated gut wasting and villous shortening (Chance et al., 1997).

Other studies have also shown that inclusion of some specific nutrients such as short-chain fatty acids (Stein et al., 1994, Tappenden 1997), glutamine (Stein et al., 1994; Burrin et al., 1994; Li et al., 1994), structured lipids ((Stein et al., 1994), and nucleotides (Kishibuchi et al., 1997; Carver and Walker, 1995) in TPN-formula can alleviate TPN-feeding associated gut atrophy and malfunction. Only a few studies have examined the effects of these adjuvants on intestinal protein metabolism in TPN-fed animals (Stein et al., 1994; Burrin et al., 1994). Although structured lipid, butyrate and glutamine suppressed the TPN-induced decrease in jejunal mucosal protein synthesis, none of the adjuvants resulted in rates of mucosal protein synthesis equal to orally fed rats (Stein et al., 1994). Cathepsins B, H, and L activities are elevated in TPN-fed animals but inclusion of nucleosides and nucleotides mixture (inosine, cytidine, uridine, thymidine, and guanosine 5-mono-phosphate) in TPN make the activities of these enzymes comparable to the levels in orally-fed animals (Kishibuchi et al., 1997). Because these studies are generally long term trials, whether the effects seen are attributable to the nutrients themselves or to nutrient-stimulated release of growth factors remains to be demonstrated.

Regulation of Protein Degradation

Studies on regulation of protein degradation in the small intestine are very limited indeed. Unlike skeletal muscle where there are reliable methods for estimating rates of intracellular protein degradation, there are as yet no satisfactory direct methods for estimating rates of protein degradation in the small intestine. One reason for this is the high rates of protein turn-over in the tissue. This makes label-recycling of high

significance, thereby complicating estimation of protein degradation. Furthermore, cell turnover in the intestinal mucosa is one of the highest in the body. After cells are produced in the crypt, they migrate to the tip of the villi from where they are shed off into the intestinal lumen along with cell-associated proteins (Alpers and Kinzie, 1973; Lipkin, 1987). The intestine loses protein, therefore, by means other than from processes of degradation. Finally, the intestinal mucosa synthesizes export proteins such as digestive enzymes, mucins, and apolipoproteins (Tygat et al., 1993; Reeds et al., 1993). In addition, incubation of intestinal cells or tissues is fraught with many complications, including cell death. All these factors make estimation of protein degradation in the small intestine difficult as methods used in other tissues for such studies are not adaptable to the intestine.

A previously used approach is the double isotope method that compares the relative rates of decay of two isotopes in a given protein (Alpers and Kinzie, 1973). One isotope (^{14}C) is given initially, allowed to decay a certain time (10 h), and then followed by another isotope (^{3}H). The animal is killed after the second isotope at an interval that is short in relation to the total decay curve. Proteins with rapid degradation rates will show a high ^{3}H: ^{14}C ratio. Using this approach it was shown that there was a correlation between molecular size and degradation rate in intestinal brush-border proteins, with larger proteins having more rapid degradation rates (see Alpers and Kinzie, 1973). This method could not however distinguish if the high turn-over of proteins was due to protein degradation or protein export.

Using stable isotope dilution technique and arteriovenous difference calculations across splanchnic tissues, Halseth et al. (1997) showed that in 18-h fasted dogs, portal-drained visceral tissues (small intestine, pancreas and spleen) contribute ~17% of whole body leucine release (protein degradation). The rate of leucine release from these tissues combined is 0.66 μmol . Kg^{-1} body weight per minute, equivalent to a proteolytic rate of 1.11 mg protein . Kg^{-1}. min^{-1}. Proteolysis was further increased 40-50% after 2 h of treadmill exercise. Because other tissues were unavoidably included in the calculation, the rate of intestinal proteolysis, as well as the contribution of intestine to whole body proteolysis remains to be determined. In addition, the techniques used in the study are not immune to the problems referred to earlier, especially the problem of label recycling.

Mechanisms involved in intestinal protein degradation are not known. Three main proteolytic systems are thought to be involved in much of the intracellular protein breakdown. These are the lysosomal, Ca^{2+}-dependent, and ATP-ubiquitin-proteasome pathways. Studies on the identification of these pathways have generally been done *in vitro* with incubated fibroblasts, skeletal muscle and liver (see Kettelhut et al., 1988 for a review). The significance of the lysosomal and Ca^{2+}-dependent pathways was identified by incubating tissues in Ca^{2+}-free media and in the presence of inhibitors of lysosomal function (for example, chloroquine, methylamine, leupeptin, or E64). For ATP-ubiquitin-proteasome system, the importance of the pathway was studied in Ca^{2+}-free incubation media containing dinitrophenol and 2-deoxyglucose to prevent oxidative phosphorylation and glycolysis.

A brief description of the three proteolytic systems is given below

Lysosomal Proteolytic System

Extracellular proteins, such as plasma lipoproteins and hormones, phagocytosed bacteria and macromolecules, undergo endocytosis and are completely degraded within lysosomes which contain several acid proteases (e.g., cysteine proteinase cathepsin B, H and L, and carboxyl (acid) protease cathepsin D) and other hydrolyses (Mitch and Goldberg, 1996; Barret and Kirschke, 1981; Takahashi and Tang, 1981; Uchiyama et al., 1994 for reviews). This endosome-lysosome proteolytic pathway is involved in the generation of peptides that are presented to the immune system on cell-surface MHC class II molecules and elicit antibody or inflammatory responses. The system also functions in the turn-over of cell-membrane proteins. It is involved in increased degradation of ligand-receptor complex following binding, leading to reduced number of receptors. Generally, lysosomal proteases have their pH optima in the acidic range and alkalinization of the lysosomes inhibit proteolysis (Mortimore and Pösö, 1987). The lysosomal proteolytic pathway can be blocked by agents that inhibit lysosomal proteases such as methylamine, chloroquine, leupeptin, pepstatin and E64 (Kettelhut et al., 1994; Uchiyama et al., 1994).

Activities of cathepsins have been demonstrated in the intestine (Healy, 1977, Dinsdale and Healy, 1982; Kishibuchi et al., 1995, 1997). Cathepsins were thought to play a role in intestinal closure (transmission of intact macromolecules such as immunoglobulins A and G from intestinal lumen across into the blood stream). It was hypothesized that because intestinal cathepsin activities increased with age, this would result in increased degradation of endocytosed proteins and prevent their intact delivery into the blood

(Davies and Messer, 1984; Brown and Moon, 1979). Although some authors found a correlation between intestinal closure and lysosomal cathepsin activities in the intestinal epithelium of neonatal rat and pig (Davies and Messer, 1984, Brown and Moon, 1979), others did not; there being no change in cathepsins B and D in 24 h, 6 d and 4-8 week old piglets (Healy, 1977, Dinsdale and Healy, 1982). Other authors have shown that the activities of cathepsins B, H and L are higher in ileal than in jejunal mucosal, and that part of the TPN-induced increase in macromolecular transmission (more noticeable in ileum), but not TPN-induced gut atrophy (more noticeable in jejunum), correlated with increased cathepsin activities (Kishibuchi et al., 1995, 1997). This was thought to be necessary so as to degrade macromolecules and bacteria that have penetrated the cell, thereby preventing their transmission into the blood.

Ca^{2+}-Dependent Proteolytic Pathway

This involves intracellular non-lysosomal calcium-activated cysteine proteinases called calpains. The proteinases have two subunits (catalytic 80 kDa and regulatory 30 kDa). The regulatory subunit is conserved in all organisms (see Johnson, 1990; Kettelhut et al., 1994 for reviews). Differences in specificity and calcium activation of different calpains subtypes result from differences in the structure of the 80 kDa catalytic subunit.

Proteolytic activities of calpains are inhibited by treatments that raise intracellular [Ca^{2+}], and by a number of endogenous small molecular weight cytoplasmic components such as isovalerylcarnitine (a metabolite of isoleucine), carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methyl histidine), and L-1-methyl-histidine. Calpastatins (specific protein

inhibitors co-expressed with calpains in most cells), peptidyl chloromethanes, peptide aldehydes, and peptidyl diazomethanes are some of the inhibitors of calpains. The polyamines spermidine and spermine have also been shown to inhibit muscle calpain II (Johnson and Hammer, 1990).

Calpains are involved in partial or complete degradation of a number of membrane proteins (such as ankyrin, spectrin, and Ca^{2+}-ATPase), and in the degradation of cytoskeletal and contractile proteins (Johnson, 1990). They have been implicated in protein degradation associated with dystrophy or damaged muscle. Treatments of normal muscle or cells that raise intracellular [Ca^{2+}] such as a reduction in ATP levels, or allowing incubated muscle to shorten *in vitro* also increased muscle proteolysis via the calpains (Kettelhut et al., 1994). Studies on the activities of calpains in intestine are very scanty. A recent study shows that after 1 or 5 d fasting in adult rats, mRNA for m-calpain increased by 151 and 94%, respectively, in the small intestine, and by ~90% in the colon, in parallel to fasting-induced protein catabolism observed in these tissues (Samuels et al., 1996).

ATP-Ubiquitin-Proteasome Proteolytic Pathway

Another proteolytic system shown to be important in intracellular proteolysis is the ATP-ubiquitin-proteasome pathway. In this pathway, ubiquitin, an 8 kDa peptide, is covalently linked via its carboxy terminus to ε-amino groups of lysine residues of target proteins. This conjugation appears to mark these proteins for recognition and degradation by the large proteolytic complex, the 26S complex, which itself contains two 19S regulatory

complexes and, as the key proteolytic component, the 20S proteasome (Wing et al., 1996; Wing and Banville 1994; see Mitch and Goldberg, 1996; Goldberg, 1995 for reviews). Before ubiquitin is conjugated to target proteins, it is first activated by ubiquitin-activating enzyme (E1) in a reaction requiring ATP, resulting in the formation of a high-energy thiol ester linkage between the carboxy terminus of ubiquitin and the cysteine residue of the active site of E1. Activated ubiquitin is then transferred by E1 via a transthiolation to the active site of cysteine of one of a family of ubiquitin-conjugating enzymes or ubiquitin-carrier proteins (E2). E2 either ligates the ubiquitin directly to proteins or requires the presence of ubiquitin-protein ligase (E3). Although E3-independent ligation does occur, E3 appears to be important in recognition and binding of protein substrates (Resiss et al., 1989). The ubiquitinated protein, unfolded by a 19S complex at one end of the core 26S proteasome, is then transferred to the central chamber of the 20S core proteasome. Here, on the proteolytic sites inside the core 20S, the proteins are cleaved progressively to peptides of 6-12 amino acids until they are degraded completely (Fig. 1- 2). These peptides are released and rapidly hydrolyzed to amino acids by cytosolic exopeptidases, except for the peptides transported to the cell surface by MHC class I molecules (Mitch and Goldberg, 1996).

Because this system involves many components, identification of the rate limiting step in the series of reactions involved is vital to understanding of the regulation of the pathway. Although ubiquitin is essential, it is thought unlikely that the peptide could be limiting as, at least in muscle, levels of ubiquitin in the fed innervated tissue appear to be saturating with E1. Additionally, because activated ubiquitin is not necessarily committed to

proteolysis, E1 is thought not to be candidate for regulation. While some authors have proposed that, at least in skeletal muscle, the tissue in which the system has been highly studied, E2 and E3 appear to catalyze the rate limiting step (Wing and Banville, 1994), this opinion is not shared by all (Attaix et al., 1998).

In skeletal muscle, various studies have shown that much of proteolysis due to denervation, starvation, sepsis, cancer, and burns is due to the activation of ATP-ubiquitin proteolytic pathway (Voisin et al., 1996; Wing and Goldberg, 1993; Kettelhut et al., 1994, Goldberg, 1995; Baracos et al., 1995; Medina et al. 1995; Mitch and Goldberg, 1996; Wing and Banville, 1994). Under these conditions, mRNA levels of components of the pathway increased in parallel with observed elevated muscle proteolysis. In small intestine and colon, a recent study shows that in parallel to proteolysis observed after 1 or 5 d of fasting, mRNA levels of ubiquitin and E2 were elevated. mRNA levels of C8 and C9 subunits of proteasome were also elevated in the colon (Samuels et al., 1996). In a different study (Hubbard and Carne, 1994) ubiquitin level was found to vary with feeding, its level being elevated by 140% after one day of fasting. Proteasome activity, as measured by chymotrypsin-like activity using Suc-Leu-Leu-Val-Tyr-MCA as a substrate, is higher in ileum than jejunum, and its activity increased in parallel with TPN-induced jejunal atrophy (Kishibuchi et al., 1995).

Although in skeletal muscle (Medina et al., 1995; Mitch and Goldberg, 1996; Voisin et al., 1996; Wing and Goldberg, 1993) and liver (Mortimore et al., 1992), ATP-ubiquitin dependent and lysosomal, respectively, are thought to be the predominant systems, the

relative importance of any of the pathways in intestinal intracellular proteolysis is not known. Studies on nutritional regulation of the various proteolytic pathways are virtually non-existent.

Because there are presently no direct methods for studying tissue protein degradation *in vivo*, one of the indirect approaches used to estimate proteolysis is the measurement of mRNA levels of components of the different intracellular proteolytic systems (Kishibuchi et al., 1995; Baracos et al., 1995; Samuels et al., 1996; Voisin et al., 1996; Wing and Goldberg, 1993; Ekstrom and Westrom, 1991). As shown in the preceding paragraphs, intestinal mRNA levels of components of the three systems are responsive to different nutritional manipulations and, especially for the ATP-ubiquitin-proteasome and Ca^{2+}-pathways, vary in parallel with intestinal protein loss observed in fasting (Samuels et al., 1996). Therefore, in addition to measurement of protein synthesis, studies that examine the effects of different nutritional manipulations on the expression of genes of components of the different proteolytic systems will enrich our understanding of intestinal protein metabolism. While this approach provides a way of estimating the effects of different nutritional manipulations on intestinal proteolysis as well as the contributions of different proteolytic pathways to such effects, the assumption is that proteolysis is regulated, at least partly, at the level of transcription. Theoretically, it is possible to observe changes in proteolysis without any changes in mRNA levels. This can occur through changes in the activities and of the amount of appropriate proteases. Changes in the amount of protease, though can result from changes in gene expression, may also result from changes in the translational process as well as changes in the rate of

degradation of the protease. Despite these limitations however, a number of studies has shown good correlation between tissue proteolysis and proteases mRNA levels (Baracos et al., 1995, Voisin et al., 1996; Wing and Goldberg, 1993).

Methodological Issues in Studying Nutritional Regulation of Protein Metabolism in the Small Intestine

As discussed in a preceding section, studies examining the effects of nutrients on intestinal protein metabolism have generally been long term feeding trials, lasting days (for example, Le Bricon et al., 1995; Li et al., 1994; Wykes et al., 1996; Yoshida et al., 1992). There are presently no systems to examine the immediate effects of nutrients per se on intestinal protein metabolism. Therefore, a system which permits the study of acute effects of luminal nutrients, and which allows a study of the effects of the route of supply of nutrients on intestinal protein metabolism is needed

Although some workers have used isolated cell preparations, intestinal epithelial cells and tissue are particularly difficult to incubate because of a number of limitations, including oxygen insufficiency, cell leakage and cell death (Watford and Krebs, 1979; Plump et al., 1987). Also, incubating epithelial cells with both the brushborder and basolateral membranes facing the same medium is unphysiological. Apart from components of plasma such as insulin and plasma proteins which are not found intraluminally, the total concentration of amino acids in intestinal lumen is several fold that of plasma in the absorptive state (intestinal lumen: 30 mM; plasma: less than 5 mM, see Adibi and Mercer, 1973; Mathews, 1991).

An alternative model is that used by Weber et al. (1989). These authors studied the effects of luminal glucose on mucosal protein synthesis by perfusing, *in situ*, a 10-cm segment of jejunum with 56 mM glucose for 1.75 h in rats. This system has the advantage of being an *in vivo* one. However, because about 30% of jejunum was perfused, plasma glucose and insulin concentrations were probably increased by the perfusion. As such, the observations made could not be ascribed to luminal glucose alone. An improvement over this system will be to perfuse, *in situ*, segments of jejunum in a larger animal in such a way that the total length of intestine perfused constitutes a small percentage of the total absorptive surface of the intestine. For instance, the length of the small intestine in a 6-week old piglet is about 10 m. Conceivably therefore, several short jejunal segments (each 10 cm in length) will constitute only a very small percentage of the intestinal absorptive surface. Perfusion of such segments with different luminal nutrients is unlikely to alter plasma concentrations of metabolites and hormones since only a small portion of intestine is perfused. This will allow systematic study of the effects of luminal nutrients on intestinal protein metabolism in a manner previously impossible. Furthermore, because the effects of four different treatments can be studied within an animal, the system will be more sensitive as individual animal variations are reduced. A similar system was used to study the effects of glutamine on endotoxin-induced intestinal permeability in piglets (Dugan and McBurney, 1995).

In vivo measurements of protein synthesis in the small intestine have generally been done by using either the continuous infusion or flooding dose approach. Although there is still

some controversy on the strengths and weaknesses of both methods (please see Garlick et al., 1994; Rennie et al., 1994), the features of the flooding dose method make it attractive for measurement of protein synthesis in the small intestine. In this method, a massive amount of the tracer amino acid (for example, phenylalanine) with high specific activity is intravenously injected into the animal. The amount injected is chosen to be several fold the intracellular free pool size of the chosen tracer amino acid. The animal is killed within 15 minutes. The principle behind this is to 'flood' all different pools of the tracer amino acid (plasma, intracellular and tRNA) so that they all have identical specific activity. This will make it appropriate to use either the plasma or the intracellular pool as the surrogate pool for the true (tRNA) protein synthesis precursor pool, which is not as easily accessed. For the intestine, the short labeling period involved will ensure that much of the protein synthesized will not have been exported, and will therefore give a better estimate of protein synthesis. This method has been used extensively to study protein synthesis in this and other tissues (Garlick et., al 1980; Burrin et al., 1991a; Attaix et al., 1992; LeBricon et al., 1995; Lo and Ney, 1996; von Allmen et al., 1992; Breuille et al., 1994; Samuels et al., 1996).

The small intestinal mucosa can incorporate amino acids from either the luminal or basolateral side into protein (Alpers, 1972; Alpers and Their, 1972). Recent studies on the effects of route of tracer administration showed that intravenous and luminal routes resulted in similar rates of protein synthesis (Weber et al., 1989; Nakshabendi et al., 1995). However, those data were obtained using a continuous infusion of a tracer for 1-4 h. For the reasons stated above, this condition may be less than ideal for tissues such as

the small intestine. If a flooding dose can be delivered luminally, such will ensure better estimation of intestinal mucosal Ks. Furthermore, the requirement for radioactively labeled amino acid could be reduced by up to 90% since only small segments of intestine would be perfused.

In measuring protein synthesis, following the administration of tracer (phenylalanine) and collection of tissue samples, it is necessary to separate phenylalanine from its metabolites, notably tyrosine, which might have become labeled due to hepatic and renal metabolism of the amino acid. One way of doing this is by enzymatic conversion of the tracer to β-phenylethylamine, a metabolite that is not normally found in animal tissues (Garlick et al., 1980). β-phenylethylamine is then extracted and its specific radioactivity is taken as reflecting that of phenylalanine. Because intestinal tissue does not catabolize phenylalanine (Goodwin, 1979, Kaufman, 1971), luminal delivery of tracer phenylalanine also saves cost and analytical time since it is not necessary to separate the tracer from its metabolites that might have become labeled due to metabolism. Therefore, a multi-segment perfusion system that combines luminal flooding dose technique for measurement of protein synthesis offers unique advantages over any system currently in use to study the acute effects of luminal nutrients on intestinal mucosal protein metabolism.

Significance of Intestinal Protein Metabolism to Human Health and Livestock Production

An *in vivo* model that allows examination of the direct effects of nutrients on intestinal metabolism, and that incorporates methods for estimating protein synthesis and degradation will greatly enhance our understanding of regulation of protein metabolism in the small intestine. Development of such methods will permit studies that will provide insights in to the role of specific nutrients, such as amino acids, glucose and lipids, on intestinal protein metabolism and growth. Such methods will also permit examination of effects of such nutrients on intestinal growth during pathological conditions, especially in TPN-associated gut wasting, and whether the roles of such nutrients depend on the routes of delivery.

Understanding of regulation of intestinal protein metabolism and growth is also important from the standpoint of livestock production. Feed given to livestock is of no use unless digested and absorbed. Enzymes and transporter proteins involved in these processes are produced in the intestine. Other physiologically important peptides and hormones such as glucagon-like peptides, glycoproteins, and immunoglobulins, all of which are vital to animal health and performance, are also produced in the intestine. Therefore protein metabolism in the small intestine is very vital to the metabolism of the whole animal, especially in young animals where potentials for growth can only be exploited if nutrients are supplied to the animal.

Scope of the Thesis

The aim of this research is to examine the acute nutritional regulation of protein metabolism in the small intestine. To achieve this, specific hypotheses and objectives of the studies described in chapters 2-4 are summarized below

Chapter 2: Although a number of nutrients has been shown to have regulatory effects on intestinal protein metabolism, previous studies utilized feeding trials and therefore could not show whether the effects of these nutrients were direct or indirect. A system that allows controlled examination of the acute effects of nutrients on intestinal metabolism is needed. The aim of the experiments described in this chapter was to validate such a system.

Hypotheses:

1. Luminal and intravenous flooding dose techniques will give identical estimates of protein synthetic rates in small intestinal mucosa;
2. Multiple jejunal segments within the same piglet will have identical rates of protein synthesis.
3. Luminal nutrients perfused through intestinal segments will not significantly affect plasma levels of perfused nutrients and insulin.

To examine these hypotheses, the **specific objectives** were to

1. Examine the effects of route of tracer administration (luminal or intravenous) on intestinal mucosal protein synthesis;

2. Examine the effects of surgical and perfusion procedure on intestinal mucosal protein synthesis;

3. Perfuse multiple (four) segments within the same piglet and examine rates of protein synthesis in the four segments;

4. Validate the multiple-segment perfusion system for studying the acute effects of luminal nutrients on intestinal protein metabolism by perfusing the segments with different nutrient solutions and examining the effects of these perfused nutrients on plasma metabolites and insulin.

Chapter 3: Intestinal mucosa is routinely presented with nutrients. Because previous studies have shown that feeding and specific feed nutrients such as proteins and amino acids increased intestinal protein synthesis, the aim of the experiments described in this chapter was to examine the acute effects of these luminal nutrients on intestinal mucosal protein metabolism. Additionally, because glutamine, glucose, short-chain fatty acids, and ketone bodies are preferentially utilized as sources of energy by intestinal epithelial cells, we examined their effects on jejunal mucosal protein synthesis.

Hypothesis:

1. Luminal amino acids and glucose will stimulate mucosal protein synthesis and suppress the expression of proteolytic genes.

2. Luminal perfusion with glutamine, glucose, short-chain fatty acids and ketone bodies will provide energy to drive protein synthesis and therefore increase mucosal protein synthesis.

Objectives:

Examine the effects of different luminal nutrient (amino acid mixture, glutamine, glucose, short-chain fatty acids, and ketone bodies) perfusion on

1. Intestinal mucosal protein synthesis
2. mRNA of proteolytic genes.
3. Mucosal intracellular free amino acid concentrations.

Chapter 4: Because the intestinal mucosa is exposed to nutrients and stimuli from both the intestinal lumen and blood, it is conceivable that the response of this tissue would be different when exposed to nutrients simultaneously from both sides, compared to the effects of nutrients from either side. In the absorptive state for instance, while the brushborder membrane is exposed to nutrients, the basolateral membrane is, in addition, exposed to insulin and other growth factors released in response to elevated plasma levels of nutrients. Although a direct effect of insulin on intestinal protein metabolism has not been examined, this hormone exerts anabolic influences on other tissues in body, especially skeletal muscle.

Therefore the aim of the study in this chapter is to examine the individual and combined effects of luminal and elevated plasma glucose and / or insulin level, on intestinal mucosal protein metabolism.

Hypothesis:

1. Elevated plasma glucose and / or insulin will increase intestinal mucosal protein synthesis and suppress the expression of proteolytic genes.

2. The stimulatory effects of elevated plasma glucose and / or insulin on intestinal mucosal protein synthesis and expression of proteolytic genes will be further enhanced by luminal perfusion of amino acids.

Objectives:

1. Examine the effects of intravenous glucose infusion (to raise both plasma glucose and insulin levels) on mucosal protein synthesis.
2. Examine the effects of luminal amino acid perfusion on mucosal protein synthesis in piglets that are infused intravenously with saline or glucose.
3. Examine the effects of intravenous and luminal nutrients (as described in 1 and 2 above) on intestinal proteolysis by measuring mRNA levels of m-calpain and of genes involved in ATP-ubiquitin-proteasome proteolytic pathway.

Table 1-1: Factors Affecting Intestinal Protein Synthesis.

Factors	Layer	Effects	Model	Ref
Nutritional Factors				
Fasting	Mucosa, serosa	-	In vivo (rat)	24, 85
Glutamine	Mucosa cells	+	In vitro, in vivo (rat)	55, 112
Short-chain fatty acids	Mucosa	+	In vivo (rat)	112
Triglycerides	Mucosa	+	In vivo (rat)	62, 112
Milk (vs. water)	Mixed	+	In vivo (pig)	26
Colostrum (vs. milk)	Mixed	+	In vivo (pig)	7, 26
Solid feed (vs. milk)	Mixed	+	In vivo (lamb, pig)	7, 26
Protein Deprivation	Mucosa	-	In vivo (rat, pig)	83, 129
Physiological Factors				
Weaning	Mixed, Mucosa, serosa	+	In vivo (rat, lamb)	7, 24
Lactation	Mixed	+	In vivo (mouse, rat, goat)	13, 14, 88
Pregnancy	Mixed	+	In vivo (rat)	88
Endocrine Factors				
Insulin	Mucosal cells	+	In vitro (rat)	95, 96
IGF-1	Mucosal cells	+	In vitro, in vivo (rat)	95, 96
	Mucosa, serosa	+	In vivo (rat)	74
Thyroid hormones	Serosa, whole	+	In vivo (rat, pig)	81, 109
Pathological Factors				
Sepsis / Endotoxin	Mucosa, serosa	+	In vivo, in vitro (rat)	20, 56
Il-1, TNF-α	Mucosa	+	In vivo (rat)	31, 120
	Serosa	0		
Others				
Ethanol	Mucosa, serosa	-	In vivo (rat)	80, 98

+ = stimulation; - = suppression; O = no effect

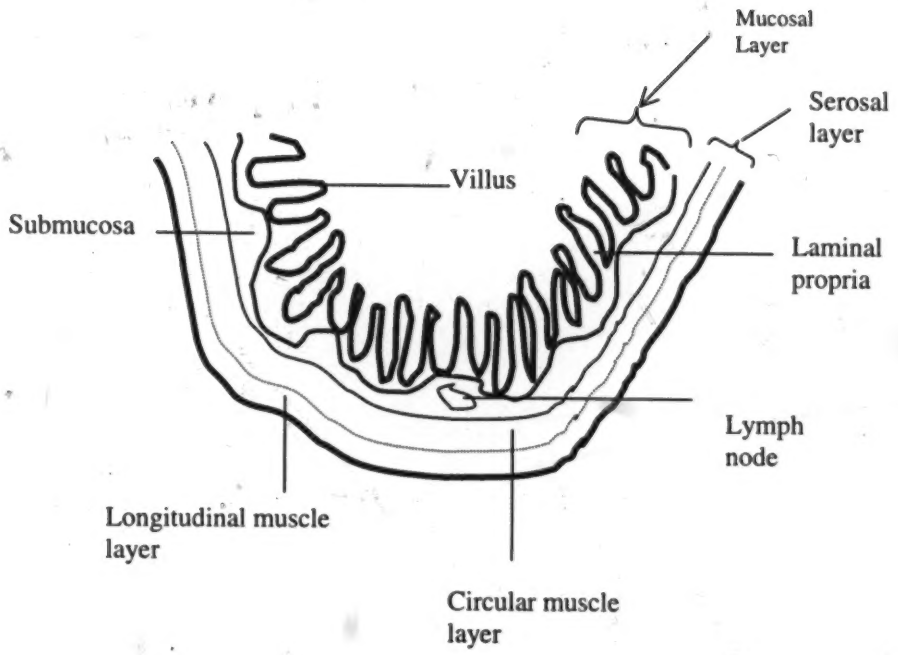

Figure 1-1. Organization of the Different Layers of the Small Intestine (Adapted from Berne and Levy, 1988).

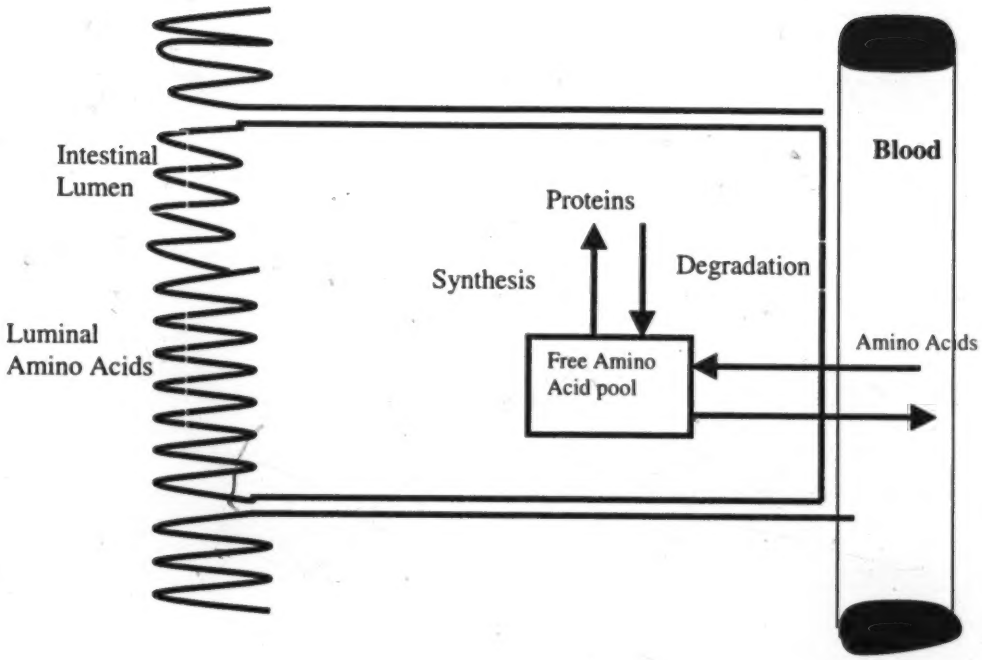

Figure 1-2. Utilization of Amino Acids from Intestinal Lumen and the Blood for Protein Synthesis by Mucosal Epithelial Cells

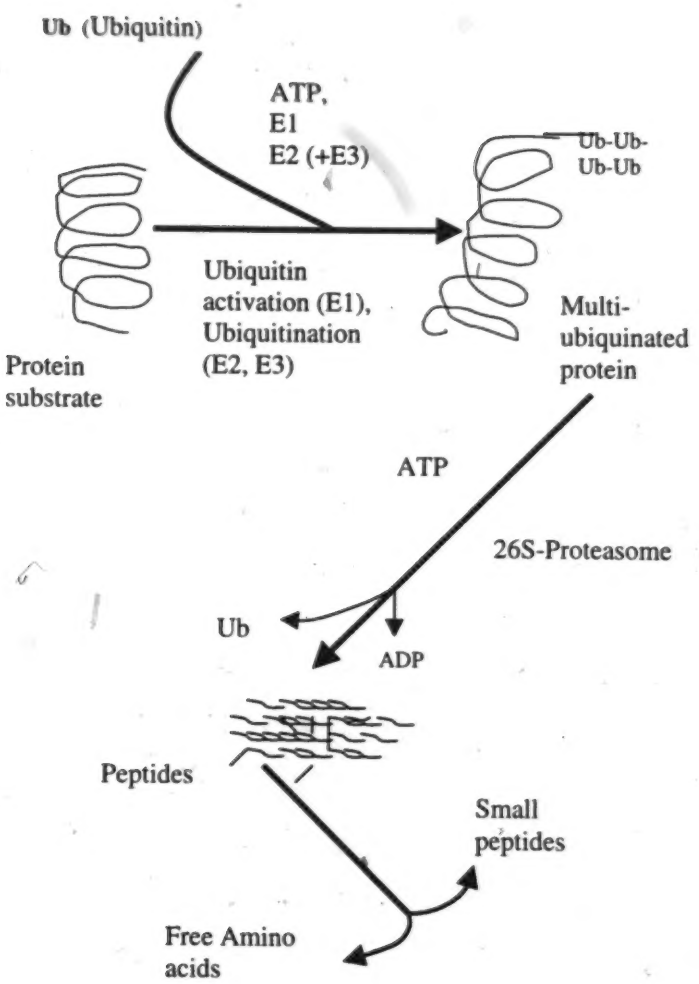

Figure 1-3. Pathway through the ATP-Ubiquitin-Proteasome Proteolytic System (Adapted from Mitch and Goldberg, 1996).

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CHAPTER 2

Jejunal Mucosal Protein Synthesis: Validation of Luminal Flooding Dose Method[1]

INTRODUCTION

The small intestine is an important component of whole body protein metabolism, accounting for up to 12% of total protein synthesized (Simon et al., 1982). Although intestinal mucosa is routinely presented with luminal nutrients, previous studies focused only on long term feeding trials and therefore do not allow distinction between the effects of nutrients and those of growth factors and hormones released in response to feeding. Thus, acute regulation of intestinal mucosal protein synthesis by luminal influences is largely unknown. Although use of isolated enterocytes has been employed (Higashiguchi et al., 1993), this system does not allow distinction of luminal versus basolateral influences. This is an important factor because the brush-border and basolateral membranes of epithelial cells of the intestinal mucosa are exposed to two different environments and regulation of metabolism can be effected through either of the two membranes.

Small intestinal mucosa can incorporate amino acids from either the luminal or basolateral side into protein (Alpers, 1972; Alpers and Their, 1972). Recent studies on the effects of route of tracer administration showed that intravenous and luminal routes resulted in similar rates of protein synthesis (Nakshabendi et al., 1995; Weber et al.,

[1] A version of this chapter has been accepted for publication. O. A. J. Adegoke, M. I. McBurney, and V. E. Baracos. Am. J. Physiol. (Gastrointest. Liver Physiol.), 1998.

1989). However, those data were obtained using a continuous infusion of a trace amount of labeled amino acid for 1-4 h. This condition may be less than ideal for tissues such as the small intestine where protein turnover is rapid (Garlick et al., 1994) and secretory proteins are produced (Smith and Moor, 1984). The features of the flooding dose technique (Garlick et al., 1980, 1994) make it a better approach for studying intestinal mucosal protein synthesis. If a flooding dose can be delivered luminally, this will be distinctively advantageous in that the requirements for radioactively labeled amino acid could be reduced by up to 90%. Furthermore, because catabolism of phenylalanine by intestinal tissue is very limited (Goodwin, 1979; Kaufman, 1971), luminal delivery of tracer phenylalanine would also save cost and analytical time since it is not necessary to separate the tracer from its metabolites that might have become labeled due to metabolism. Finally, simultaneous but independent perfusion of multiple jejunal segments within an animal would permit compound observations within an animal.

Change in cellular hydration state, resulting from exposure to anisosmotic environments, nutrients, or hormones, is one of the mechanisms involved in regulation of cellular metabolism (Busch et al., 1996; Häussinger, 1996). For example, exposure of hepatocytes to hypo-osmotic environment induces variations in hydration states. Such variations trigger anabolic an cascade of events, including alkalinization of the lysozomes, leading to decreased proteolysis and increased protein synthesis (Busch et al., 1996; Häussinger, 1996). Although intestinal mucosa is exposed to variability in luminal osmolarity (Ferraris et al., 1990), the effect of modest changes in luminal osmolarity on intestinal protein metabolism is not known.

The objective of this work was to validate a system that could allow a systematic study of the acute effects of luminal nutrients on intestinal mucosal protein synthesis. Apart from the advantages of an *in vivo* system, variability is reduced as the effects of four luminal treatments can be tested within the same animal. We examined the suitability of luminal flooding dose technique to measure jejunal mucosal protein synthesis and determined the effects of surgical / perfusion procedures and associated trauma on protein synthesis in jejunal mucosa. We also compared rates of protein synthesis in multiple jejunal segments within an animal using the luminal flooding dose technique. It is essential that this system be functionally a first pass system for it to be useful. Therefore the effects of luminal perfusion with different nutrient solutions on systemic parameters were also determined. A final objective was to examine the effects of physiological variations in luminal osmolarity on jejunal mucosal protein synthesis.

MATERIALS AND METHODS

Chemicals

L-[2,6-^{3}H] phenylalanine was purchased from Amersham International (Amersham Place, Little Chalfont Bucks UK). L-Tyrosine decarboxylase, pyridoxal phosphate, phenylalanine and β-phenylethylamine were from Sigma Chemical (St. Louis, MO). Tubing for cannulation was from Fisher Scientific Company, PA.

Animals, Surgical and Perfusion Procedures

All experiments were performed in accordance with the Canadian Council on Animal Care Guidelines. Male, Sprague-Dawley rats (100-170g) and 6-week old piglets were

obtained through the University of Alberta Health Sciences Laboratory Animal Services. The piglets were weaned at four weeks and maintained on a standard starter diet (crude protein 20.46%, digestible energy, 3.55 kcal/kg) while rats were offered laboratory chow. Prior to experiments, animals were fasted overnight but water was made available at all times.

Rats were maintained under anesthesia with 1.5-2.5 % halothane delivered with oxygen. A 10-cm segment of the jejunum was cannulated at both ends with polyethylene tubing (ID = 1/16"; OD = 1/8"). Feed remnants in the segment were cleaned out with warm phosphate-buffered saline (PBS, 144.6 mM NaCl, 15.9 mM Na_2HPO_4 1.2 mM, $NaH_2PO_4.H_2O$, pH 7.4, 37°C).

Anesthesia was induced in piglets with a mixture of Torbugesic (0.2 mg/kg), Ketamine (11 mg/kg), Rompun (2.2 mg/kg) and glycopyrrolate (0.01 mg/kg), and maintained with 2% halothane delivered with oxygen. Four 10-cm jejunal segments were cannulated at both ends with polyethylene tubing (tube diameters: ID = 1/8 and 3/32", OD = 3/16 and 5/16", respectively for inlet and outlet cannulas). Inlet cannula for the first segment was inserted 15cm from the ligament of treitz while successive sections were separated by 50 cm of intestine (see Plate 2-1). Segments were rinsed of digesta remnants with warm PBS. A similar preparation has previously been used to study glutamine metabolism in endotoxin-treated piglets (Dugan and McBurney, 1995).

Experiments

Comparison of Conventional and Luminal Flooding Dose Techniques

For measurement of protein synthesis with luminal delivery of tracer, the perfusion solution was 2 mM [2,6-^{3}H]phenylalanine, specific radioactivity, sr = 730 dpm / nmol. This concentration was chosen because a preliminary study showed that under intravenous flooding conditions in rats, plasma concentrations of phenylalanine did not rise beyond 2 mM. Also, when a 10-cm jejunal segment of pig or rat were perfused (re-circulated) with 2 mM phenylalanine for 15 or 30 minutes, the concentration of the tracer did not change appreciably. The observed values (mean ± standard deviation, n = 2 for each species) are for rat: initial amount of phenylalanine in perfusate = 19.4 ± 2 µmol, amount remaining after 15 minutes of perfusion = 19.6 ± 1 µmol; pig: initial amount of phenylalanine = 70.3 ± 8 mmol, amount remaining after 30 minute perfusion = 67.6 ± 13. This was presumably because large volumes, relative to the volume of the segments, were perfused (volume of perfused solution 10 and 50 mL; volume of intestinal segments: 0.7 and 5 mL, for rat and pig perfusion, respectively).

To examine the effect of route of tracer delivery on protein synthesis, one group of rats was luminally perfused (1 mL / min) with tracer solution for 2 or 10 minutes (luminal-flood, n = 6-7 for each time point). A second group of rats (iv-flood, n = 5 for each time point) received only a standard intravenous flooding dose (150 mM L-[2,6-^{3}H] phenylalanine, sr = 730 dpm/nmol, 1 mL/100g body weight (12)). To examine the effects of perfusion procedures on protein synthesis, a third group of rats (iv+perfusion, n=7-8 for each time point) underwent surgery and intestinal cannulation and perfusion with PBS. These rats also received an intravenous flooding dose of tracer as done for iv-

flood' rats. Tissues were harvested after 2 or 10 minutes. Intestinal segment was cut open longitudinally and then spread on a cold surface with the mucosal side facing up. Mucosal samples were frozen in liquid N_2 and stored at -40°C.

Mucosal Protein Synthesis in Multiple Jejunal Segments and Effects of Cannulation and Perfusion Procedures

Four jejunal segments within each piglet (n = 7) cannulated as described above were individually but separately perfused with luminal flooding dose as described for rats but for 15 minutes at 6 mL / min. Mucosa from these segments were stored frozen as described for rats. To examine the effect of extended perfusion time on basal rate of protein synthesis, two intestinal segments within the same piglet (n = 2) were separately but simultaneously perfused with PBS for 1.5 h. Afterwards, animals were injected, via the jugular vein, flooding dose of tracer (150mM L-[2,6-^{3}H]phenylalanine, specific radioactivity = 730 dpm/nmol at 8 mL/kg body weight). Mucosal samples from perfused and adjacent unperfused segments were collected 15 minutes after isotope injection and stored frozen.

Systemic Effects

For a system employing multiple segments within an animal to be useful, the absence of systemic alterations after application of luminal treatments must be documented. If luminal perfusion of multiple jejunal segments with nutrient solutions did not raise systemic levels of perfused nutrients or hormones, then the treatment could be considered first pass nutrients. This was examined in two ways. In animals administered tracer luminally, the only source of radioactivity in adjacent, unperfused segments, is the

absorbed tracer that has been re-distributed systemically. Therefore, determination of phenylalanine sr in this segment is useful in assessing the effects of luminal perfusion on systemic parameters. Secondly, intestinal segments within overnight-fasted piglets (n=3) were independently but simultaneously perfused for 1 h with: (i) 30 mM glutamine, (ii) 30 mM amino acid mixture plus 50 mM glucose, (iii) same as (ii). The amino acid mixture contained (mM) aspartate (0.67), serine (2.03), glutamate (2.34), glutamine (1.84), proline (3.15), glycine (3.75), alanine (2), cystine (0.3), tyrosine (0.79), histidine (0.59), arginine (1.29), asparagine (0.73), threonine (1.34), valine (1.66), methionine (0.55), isoleucine (1.14), leucine (2.09), phenylalanine (2), lysine (1.58), and tryptophan (0.19). These concentrations were based on observed luminal total free amino acid and glucose levels after a meal in rat, rabbit and pig (Adibi and Mercer, 1973; Ferraris et al., 1990; Low, 1979).

Blood samples were taken before and at the end of perfusion. Plasma was stored frozen at -40^{0}C until analyzed for glucose, amino acids, and insulin concentrations.

Effects of Luminal Osmolarity on Protein Synthesis in Intestinal Mucosa

One of 4 jejunal segments within the same piglet (n = 4) was perfused with PBS, pH 7.4, containing 250, 300, 339 and 380 mosmoles/L for 1.5 h. Segments were then emptied and perfused for 15 minutes with the same solution but containing 2 mM L-[2,6-^{3}H] phenylalanine, specific radioactivity = 730 dpm/nmol. Mucosal samples were collected and treated as described above.

Sample Processing and Analysis

Determinations of phenylalanine sr in plasma, and in mucosal free and protein-bound fractions were done by conversion of phenylalanine to β-phenylethylamine as described previously (Garlick et al., 1980; Samuels and Baracos, 1995). This is because with intravenous tracer administration, phenylalanine radioactivity can be transferred to other metabolites, especially tyrosine, due to hepatic and renal metabolism of the tracer (Goodwin, 1979; Kaufman, 1971), leading to over-estimation of sr.

Since catabolism of phenylalanine by the intestinal mucosa is very limited (Goodwin, 1979; Kaufman, 1971), radioactivity measured in luminally flooded intestinal segments should be associated mainly with phenylalanine. To examine this, mucosal samples from luminally flooded segments were analyzed for free and protein-bound total phenylalanine and radioactivity with or without conversion of phenylalanine to β-phenylethylamine. After accounting for the efficiency of the enzymatic conversion and of extraction of β-phenylethylamine, we established that the entire measured radioactivity was accounted for by phenylalanine. Thus, in piglets studies involving luminal flooding, phenylalanine specific radioactivity was calculated without conversion of phenylalanine to β-phenylethylamine.

Fractional rate of protein synthesis (Ks), expressed as % per day, was calculated according to Garlick et al. (1980),

$$Ks = 100\, S_b / S_f\, t$$

where S_b is the specific radioactivity of protein-bound phenylalanine, t is the time (in days) between injection and slaughter (or duration of luminal isotope perfusion), S_f is the mean intracellular free phenylalanine sr between 2 and 10 minutes in rat studies. In

piglet experiments, S_f is the intracellular free phenylalanine sr in tissue samples collected terminally after it was shown that this variable did not change for up to 40 minutes of isotope perfusion (see Result section).

Plasma glucose concentration was measured using Glucose (Trinider) Kits (Sigma Diagnostics, St. Louis, MO) while plasma insulin concentration was determined using Enzymun-T Insulin Assay (Boehringer Mannheim Immunodiagnostics, Laval Quebec, Canada). Amino acid concentrations were determined by high performance liquid chromatography (Strelkov et al., 1989).

Data are expressed as means ± SE and were analyzed by analysis of variance. Differences amongst means were examined using Fishers Protected Least Significant Difference using SAS statistical package (1982).

RESULTS

Comparison of Conventional and Luminal Flooding Dose Techniques

Plasma and mucosal free phenylalanine sr were lower at ten minutes compared to the values at two minutes with intravenous tracer administration (Fig. 2-1, $P<0.05$). Although free phenylalanine sr in mucosa of luminally flooded rats was slightly lower than that observed in the intravenous groups, it is noteworthy that mucosal free phenylalanine sr in the luminal-flood group remained steady at the two time points studied. Routes of flooding or perfusion procedures had no effect on mucosal Ks (Fig. 2-2).

Mucosal Protein Synthesis in Multiple Segments of Jejunum

In multiple segments constructed within same piglet, mucosal free phenylalanine sr and Ks were similar (Fig. 2-3, P>0.05). Since terminal intracellular free phenylalanine sr was used in the calculation of Ks, it was necessary to examine changes in free phenylalanine sr over the period of measurement. One piglet was luminally perfused with labeled phenylalanine for 13, 23, 31 and 40 minutes. Similar to rat data (Fig. 2-1), free phenylalanine sr stayed constant throughout, being 492, 494, 486 and 497 dpm/nmol, respectively, at the four time points. Because intestinal mucosa synthesizes export proteins, we examined the amount of protein secreted into the perfused segments during the time of label incorporation. To do this, the entire perfusate was collected after fifteen minutes of perfusion and assayed for protein concentration. The total amount of proteins in perfusate (cell-associated and free proteins) was 2.06 mg. Typically, we got approximately 500-700 mg of mucosa per perfused segment. Assuming the mucosa is 10 % protein (McNurlan et al., 1979), the maximum amount of secreted protein within the time of isotope perfusion would be 3.4% of total mucosal protein.

Effect of Cannulation and Extended Perfusion Time on Mucosal Protein Synthesis

After perfusing segments of intestine with PBS for 1.5 h, mucosal Ks, measured with the intravenous flooding dose technique, was identical to that obtained in mucosa of adjacent jejunal segments that had not been perfused (Fig. 2-4, P > 0.05). Because we did not correct for any decline in plasma free phenylalanine sr over the labeling period, the rate of protein synthesis obtained in these two piglets might have been overestimated. This however would not affect the conclusion drawn from the experiment since comparison is within piglets. Additionally, the Ks values obtained are comparable

to those in Fig. 2-3. Thus, as in rat experiments, the route of flooding did not affect the estimate of piglet jejunal mucosal Ks.

Systemic Effects

Compared to segments luminally perfused with radioactive tracer, free phenylalanine sr in plasma (28 ± 6 dpm / nmol) and in mucosa of adjacent jejunal tissue not perfused with isotope (36 ± 7 dpm / nmol) were negligible, being only 5-7% of free phenylalanine sr in mucosa of luminally flooded segments (516 ± 6 dpm / nmol, n = 4, P = 0.0002). Also, protein-bound phenylalanine sr in mucosa of adjacent unperfused jejunal tissue (0.17 ± 0.15 dpm / nmol) was only 5% of the corresponding values in luminally flooded segments (3.18 ± 0.15 dpm / nmol, n = 4, P = 0.0002). Plasma phenylalanine concentrations before and after perfusing four jejunal segments within a piglet with 2 mM phenylalanine were identical (0.133 ± 0.01 vs. 0.143 ± 0.01 μmol/mL, n=2, P>0.05). Thus luminal flooding with phenylalanine in up to 4 intestinal segments had minimal effects on tracer plasma concentration, and on its specific radioactivity in plasma and adjacent unperfused intestinal segments.

To further test for systemic changes resulting from luminal nutrient perfusion, we examined plasma variables in animals in which all segments were perfused with PBS, versus animals with segments perfused with high concentrations of glucose (50 mM) and amino acids (30 mM), or glutamine (30 mM). Plasma glucose concentrations were similar before and at the end of perfusion (Table 2-1). Insulin levels at both times were below detection limit (4 μU/mL). This is not unusual as the piglets were fasted for 16 h before the experiment. Plasma concentrations of individual amino acids were also not

affected by luminal nutrient perfusion. The exceptions to these are the concentrations of valine, glycine and alanine, which were 33-40% higher in the amino acid-perfused group, compared to the saline-perfused piglets (Table 2-1). Thus under the conditions of the experiments, luminal perfusion with glucose or / and an amino acid-rich mixture had minimal effects on circulating levels of glucose, insulin, and of most amino acids.

Osmolarity and Protein synthesis in Jejunal Mucosa

Luminal osmolarity in the range 250-380 mosmol/L did not affect tissue free phenylalanine sr (618 ± 15, 611 ± 15, 591 ± 11, 594 ± 6 dpm/nmol, $P>0.05$) or Ks (Fig. 2-5, $P>0.05$).

DISCUSSION.

We have described and validated a system for studying the acute effects of luminal nutrients on intestinal mucosal protein synthesis. Mucosal Ks determined after luminal or intravenous tracer administration were identical. Using luminal administration of flooding dose of tracer, multiple jejunal segments constructed within the same animal had similar mucosal free phenylalanine sr and Ks. This implies that each of the four intestinal segments constructed within a piglet can serve as an experimental unit so that the effects of different luminal treatments on mucosal protein synthesis can be tested, *in vivo*, on the same piglet. Also, we found out that when the intestinal lumen was the sole source of tracer, administered tracer radioactivity was associated mainly with phenylalanine, indicating that the tracer was not metabolized to other compounds by the

intestinal tissue. Thus, cost, time, and analytical error, associated with isolation of phenylalanine from any other metabolites that might have become labeled, are removed. As to be expected from the percentage of intestine that was perfused with ^{3}H-tracer, labeling of plasma phenylalanine was very limited. In piglets of the size used in these experiments, the small intestine is at least 10 m long. Since we perfused a total length of 40 cm, only 4% of small intestine was exposed to radioactive tracer. For comparison with rat studies, it is important to note that the 10-cm segment perfused constitutes about 13% of small intestinal length. Our observations do not imply that absorption of phenylalanine (or any other amino acids) is impaired. Rather, because intestinal surface area perfused in the piglet was small relative to the total absorptive capacity, the relatively small amount of absorbed amino acids do not affect plasma concentration and specific radioactivity appreciably. Other factors that might have contributed to low plasma specific radioactivity include loss of phenylalanine-associated radioactivity due to hepatic metabolism of the tracer, and dilution of absorbed tracer by unlabeled phenylalanine resulting from high protein turn over of portal-drained visceral tissues (Nakshabendi et al., 1995).

In luminally flooded rats, we also observed that intracellular free phenylalanine sr was lower at two minutes compared to the values in intravenously flooded groups. One reason for this might be the unstirred water layer effect (Mathews, 1991), which would make phenylalanine concentration at brushborder membrane lower than that present luminally, therefore reducing the efficiency of the flooding. Apart from the reason given in Materials and Methods for the choice of phenylalanine concentration of 2 mM, we did not wish to use an unphysiological concentration, as this is one of the main criticisms of

the intravenous flooding dose (Rennie et al., 1994). Two millimolar is close to the luminal phenylalanine concentration after a protein meal (Adibi and Mercer, 1973, Mathews, 1991). A second possible reason for the observed lower free phenylalanine sr in luminally flooded intestine may relate to the fate of absorbed amino acids in the intestinal mucosa. Amino acids absorbed basolaterally can either be incorporated into protein, or metabolized to other compounds. For amino acids absorbed luminally however, a third route of disappearance would be the release of absorbed amino acids into the portal circulation. Finally, differences in Km for phenylalanine transport at the brush border and basolateral membranes might also have contributed to this observation (Basolateral K_m = 0.18 mM, Brushborder K_m = 2.7 – 4.7 mM, see Hu and Borchadt, 1992). Because of these reasons, it might be easier to flood through the basolateral than the brush-border membranes. Our observation that phenylalanine specific radioactivity in perfused segments reached 500 dpm / nmol within two minutes of onset of perfusion (Fig. 1) clearly demonstrated that absorption was not impaired in the studied segments.

It was necessary to test whether luminal perfusion with nutrient solutions affected systemic levels of the perfused nutrients, and whether the surgical and perfusion procedures affected intestinal mucosal protein synthesis. Because the perfused segments constitute only about 4% of the absorptive surface area of the small intestine, and because perfusion lasted only 60 minutes, luminal perfusion of jejunal segments with nutrient solution did not alter plasma concentrations of the perfused glucose, amino acids or insulin. Only a small rise in the plasma concentrations of alanine, glycine, and valine was attributable to luminal perfusion. There was, however, an effect of surgery/ anesthesia per se on plasma amino acid levels. Previous studies have shown that surgery and anesthesia

increased plasma concentrations of essential amino acids by up to 45% when measurements were made during and immediately (2 to 12 h) post surgery (Carli et al., 1993; Lombadini and Bricker, 1981; Obata et al., 1993). Levels of non-essential amino acids, especially of alanine, glutamine, and asparagine either were unchanged or increased by 20-30%. Thus, surgery- and anesthesia-induced increases in plasma amino acid concentrations seen in this study are consistent with the literature.

It is not clear why only plasma concentrations of valine, glycine and alanine were increased by the luminal perfusate, more so when the concentration of leucine in the perfusate was higher than that of valine. Because 20 cm of intestine was perfused with 30 mM amino acid mixture for 1 h, it is possible that these few and small effects could be further reduced by perfusing only one intestinal segment (10 cm) with amino acid solution for a shorter time.

Similar to what was observed for plasma nutrient concentrations, luminal nutrient perfusion did not affect plasma insulin concentration. Insulin-like growth factor 1 and proglucagon-derived peptides such as glucagon-like peptides-1 and -2 (GLP-1 and GLP-2) are some of the gut-derived hormones implicated in the regulation of gut growth (Drucker, 1997). We did not examine the effects of luminal nutrient perfusion on gut hormone release. However, GLP-1 is insulinotropic and the anticatabolic effect of the peptide is insulin-dependent (Shalev et al., 1997). Therefore, a lack of effect of luminal nutrient perfusion on plasma insulin level would seem to imply that the amount, if any, of proglucagon-derived peptides produced are insufficient to affect systemic levels of these peptides and therefore of insulin.

Surgery does not affect whole jejunal (Bakic et al., 1988; Preedy et al., 1988) or suppresses jejunal seromuscular (Marway and Preedy, 1994) protein synthesis, whether measurements were made immediately, or two to seven days after surgery and anesthesia. These studies seem to indicate that effects of surgery on intestinal protein synthesis appear to be limited to the structural component of the tissue. However, the effect of surgical procedure on mucosal protein synthesis has not been previously reported. Our results indicate that trauma associated with intestinal cannulation and perfusion for up to 2h had no effect on jejunal mucosal protein synthesis compared to unperfused segments *in situ*. Surgery and anesthesia may however affect other intestinal parameters. Surgical bowel manipulation and anesthesia have been reported to disturb or abolish intestinal motility pattern (Sjövall et al., 1990) and to decrease blood flow to the intestine (Colombato et al., 1990). The effect of anesthesia on intestinal blood flow appears to be dependent on the type of anesthetic agent. Yano and Takaori (1994) have shown that, unlike enflurane, isoflurane does not decrease blood flow to the intestine.

Because perfusion of multiple intestinal segments within an animal had minimal effects on circulating levels of the perfused nutrients and insulin, this model permits controlled studies of the effects of nutrients in the first pass (i.e., apical but not basolateral exposure to nutrients) on intestinal protein metabolism. Moreover, because it is an *in vivo* system, many of the complications associated with incubation of isolated epithelial cells (Watford et al., 1979) are also avoided. Weber et al. (1989) used a similar preparation in the rat to study the effect of luminal jejunal perfusion with 56mM glucose for 1.75 h on mucosal protein synthesis. A major improvement of the system described here is that because of the size of the perfused segments relative to the total absorptive capacity of

the animal, systemic levels of perfused nutrients and plasma levels of hormone were not modified. Therefore, observations made could only be attributed to first-pass nutrients. The same might not be true if similar studies were to be conducted in smaller animals like rat, since the perfused segment would constitute a greater proportion of the small intestine. Perfusion of 10-cm length of rat jejunum with 56 mM glucose for 1.75 h, as done by Weber et al. (1989) would likely raise plasma glucose and insulin levels, and therefore make interpretation as to luminal *vs* basolateral, as well as nutrient *vs* hormone effects, difficult.

Cellular hydration state participates in the regulation of cell function (Busch et al., 1996; Häussinger, 1996). In hepatocytes, cell swelling increased protein synthesis and suppressed proteolysis while cell shrinking has opposite effects (Busch et al., 1996). Furthermore, the effects of hormones and amino acids on hepatic protein synthesis and proteolysis can be mimicked quantitatively when the cell volume changes that occur in response to these effectors are induced to the same degree by anisosmotic exposure (Häussinger, 1996). Following from the work of Ferraris *et al.* (1990) which showed that rat small intestinal luminal osmolality fluctuated between 316 and 384 mosmoles/kg, we examined the effects of physiological changes (250-380mosmoles/L) in luminal osmolarity on intestinal mucosal protein synthesis. Free phenylalanine specific radioactivity and protein synthesis were identical in all osmolar treatment groups. This contrasts with a previous work in which 30mM short-chain fatty acids (SFCA) and isoosmolar (NaCl) solution suppressed protein synthesis in isolated rat colonocytes (Marsman and McBurney, 1996). The differences in the design of the two experiments (species, cell and tissue types) do not allow easy comparison. Also in the study of

Marsman and McBurney (1995), isolated epithelial cells were used in contrast to the present experiment where epithelial cells retained a basolateral and brushborder sidedness and the intestinal architecture was intact.

In conclusion, this work validated the luminal flooding dose technique for studying of intestinal mucosal protein synthesis. When combined with the multiple segments perfusion system within an animal, the model offers a unique technique to study acute luminal nutrient modulation of protein metabolism in small intestinal mucosa. It allows systematic study of luminal vs basolateral effect, reduces inter-animal variability and reduces cost of experimentation. We have also used the model to show that unlike hepatocytes, the intestinal mucosa is resistant to physiological variations in luminal osmolarity with respect to protein synthesis. Ongoing studies in our laboratory are employing the model described here to examine the effects of first-pass (luminal) nutrients, as well as the route of delivery of nutrients (enteral vs parenteral), on intestinal mucosal protein metabolism.

Table 2-1: Effects of Luminal Nutrients or Saline Perfusion on Systemic Parameters[a]

	Pre-perfusion	Amino Acid Perfusion	PBS Perfusion
Amino Acids (nmol / mL)			
Total Amino Acids	4048 ± 224[b]	7224 ± 342[c]	6138 ± 296[d]
Phenylalanine	64 ± 4[b]	99 ± 5[c]	109 ± 13[c]
Valine	159 ± 15[b]	316 ± 23[c]	229 ± 20[d]
Alanine	722 ± 58[b]	1196 ± 88[c]	899 ± 2[d]
Glycine	1208 ± 104[b]	2270 ± 158[c]	1650 ± 137[d]
Total Amino Acids less			
Val, Ala, and Gly	1959 ± 131[b]	3443 ± 201[c]	3360 ± 174[c]
Glucose, mM	5.2 ± 0.3	5.8 ± 0.4	Not determined
Insulin[e], μU / mL	Undetectable	Undetectable	Not determined

[a]Values are means ± SE of plasma concentration before or after intestinal perfusion. In Saline Perfusion, four intestinal segments within the same piglet (n = 4) were perfused with phosphate-buffered saline, pH 7.4. For amino acid perfusion group (n = 3), one of four segments within the same animal was perfused for 1 h with either PBS or glutamine (30 mM), while each of the other two segments was perfused with a mixture of the 20 amino acids at proportions found in piglet digesta (30 mM total concentration) + glucose (50 mM). [bcd]Means with different superscripts within a row are significantly different (P = 0.02). Note that the effect of luminal nutrient perfusion, vs PBS perfusion, on plasma total amino acid concentration is totally accounted for by the differences in plasma concentrations of alanine, glycine, and valine. [e]Assay sensitivity, 4 μU/mL.

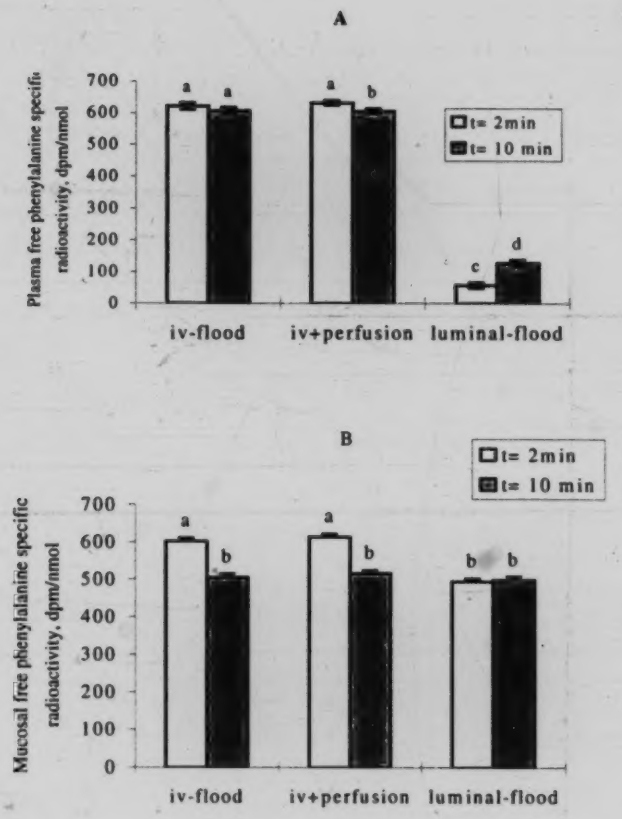

Figure 2-1. Free Phenylalanine Specific Radioactivities in Rat Plasma (A) and Jejunal Mucosa (B).

Rats were injected with 150 mmol of L-[2,6-^{3}H]phenylalanine / 100g body weight with (iv+perfusion, n = 7 or 8 for each time point) or without (iv-flood, n = 5 for each time point) luminal perfusion with PBS, pH 7.4, as described in Materials and Methods. In luminal-flood group (n = 6 or 7), rats were perfused with 2mM solution of labeled tracer. Rats were killed after two or ten minutes. Mucosal samples were processed as described in Materials and methods. Within a figure, bars (means ± SE) with different superscripts are significantly different from one another ($P<0.05$).

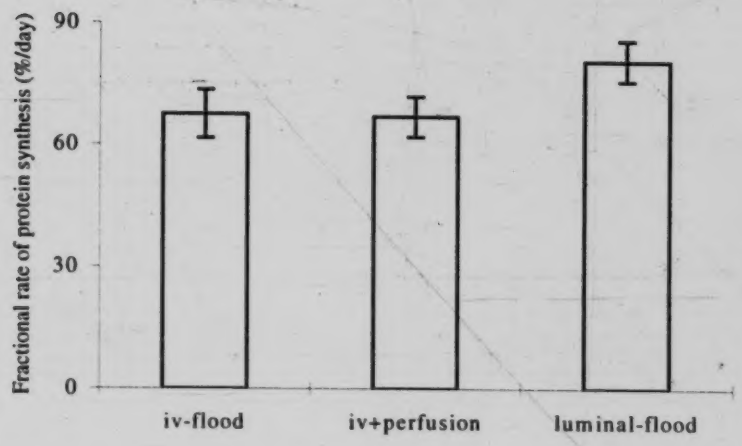

Figure 2-2. Rat Jejunal Mucosal Fractional Rates of Protein Synthesis.

Rats were injected with 150 mmol of L-[2,6-^{3}H]phenylalanine / 100g body weight with (iv+perfusion) or without (iv-flood) luminal perfusion with PBS, pH 7.4, as described in the Method section. In luminal-flood group, rats were perfused with 2 mM solution of labeled phenylalanine as described in Materials and Methods. Mucosal samples were processed as described in Materials and Methods. Data are for rats killed at 10 minutes after isotope injection or perfusion.

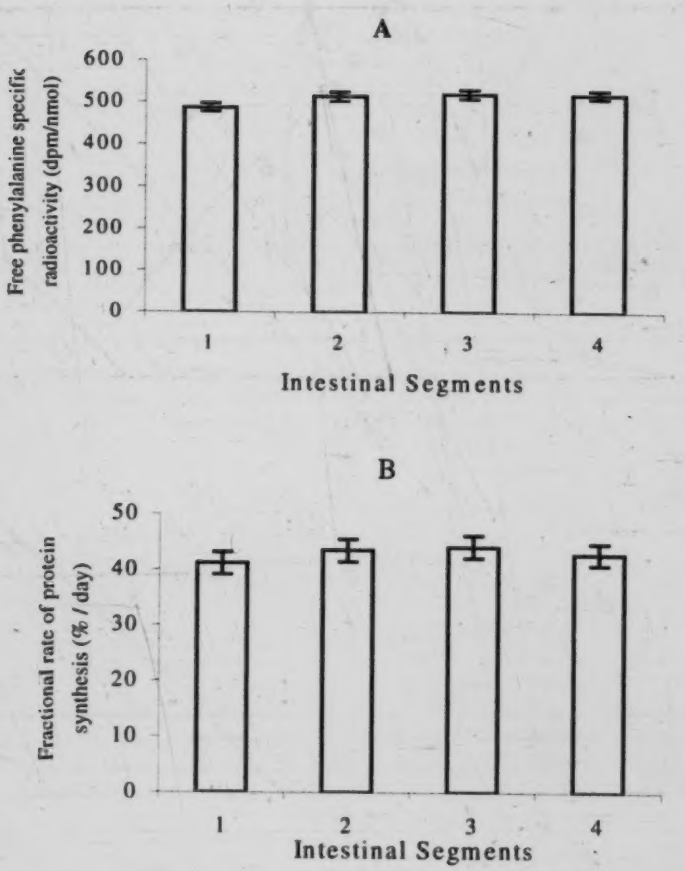

Figure 2-3. Free Phenylalanine Specific Radioactivity (A) and Ks (B) in Piglet Multiple Jejunal Segments.

Four jejunal segments within each animal (n=7), were perfused separately but simultaneously with radioactive phenylalanine solution in a re-circulating manner for 15 minutes. Mucosal samples were processed as described under Material and Methods.

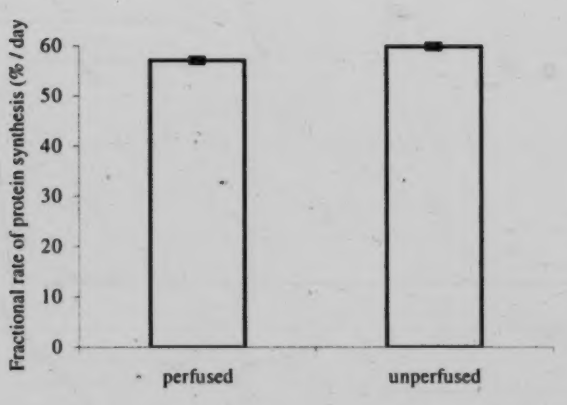

Figure 2-4. Effects of Perfusion Procedure on Piglet Jejunal Mucosal Protein Synthesis. Two jejunal segments were perfused with PBS, pH 7.4 for 1.5 h. At the end of this perfusion, a flooding dose of L-[2,6-^{3}H]phenylalanine was administered through the jugular vein. Animals were killed 15 minutes from the start of injection of isotope. Mucosal samples were taken from perfused segments and from sections of jejunum that were not perfused and processed as described under Material and Methods.

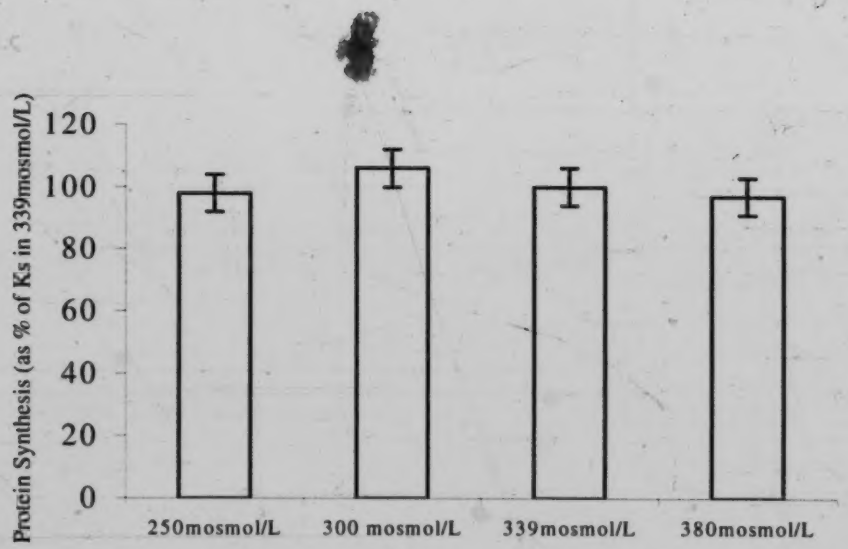

Figure 2-5. Effects of Perfusate Osmolarities on Mucosal Protein Synthesis.

One of four intestinal segments within the same piglet was perfused with phosphate buffered saline (PBS, pH 7.4) with different osmolarites for 1.5 h. Protein synthesis was then measured with luminal flooding dose as described under Materials and Methods.

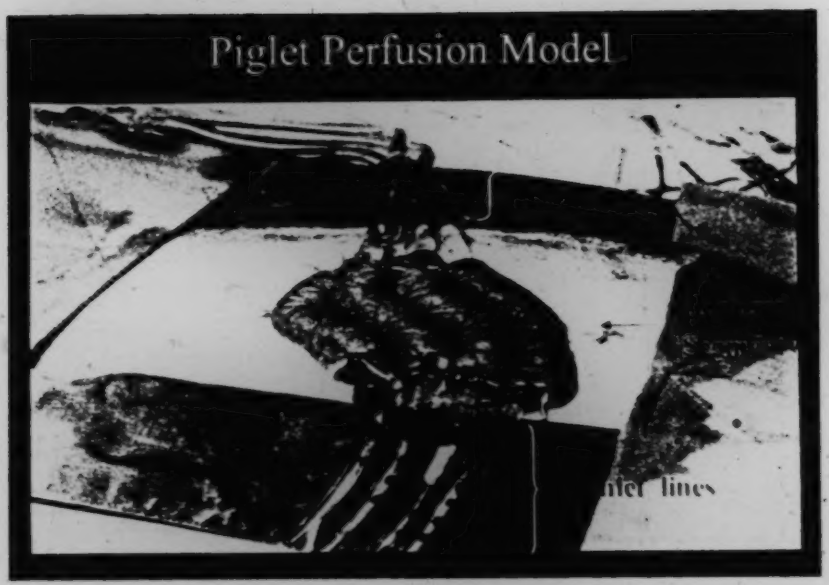

Plate 2-1. Piglet Intestinal Perfusion System.

Four intestinal segments were cannulated within a piglet. The picture shows each segment with its own inlet and outlet lines. During perfusion, the segments were covered with surgical gauze soaked in phosphate buffered saline (PBS, pH 7.4), and with transparent polyethylene sheets to reduce the rate of evaporation. The segments were additionally routinely irrigated with PBS (37°C) throughout the perfusion.

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CHAPTER 3

Acute Regulation of Mucosal Protein Synthesis by Luminal Nutrients

INTRODUCTION

The small intestine is generally less than 4% of body weight but it contributes 9-12% of daily whole body protein synthesis (Attaix and Arnal, 1987; Simon et al., 1982). Intestinal protein metabolism is regulated by nutritional manipulations. Compared to fed controls, fasting for 10 hours to five days decreased mucosal or whole intestinal protein synthesis (Ks) by 20-30% (McNurlan et al., 1979; Burrin et al., 1991; Samuels et al., 1996). Dudley et al. (1998) showed that, compared to enteral feeding, total parenteral nutrition suppressed both fractional and absolute rates of mucosal protein synthesis in neonatal piglets by about 30-40%. Feeding protein-restricted diets for 1-8 weeks also led to a fall in mucosal Ks (McNurlan and Garlick, 1981; Wykes et al., 1996). Studies with glutamine administered in total parenteral nutrition showed that this amino acid enhanced weight, DNA content, villous height and protein synthesis of the small intestine, relative to glycine or alanine (O'Dwyer et al., 1989; Stein et al., 1994). Together these studies show the importance of nutrition, of specific nutrients, and of route of supply of nutrients on intestinal protein metabolism. However, the studies cited above were generally long term feeding trials lasting days or weeks and therefore do not allow distinction of the effects of luminal nutrient exposure as separated from the contribution of systemic nutrients and / or hormones and growth factors released consequent to nutrient

consumption. Thus, the importance of nutrients in acutely regulating intestinal protein metabolism is unknown.

We recently described a perfusion system that allows study of the effects of nutrients in the first pass on intestinal protein metabolism (Chapter 2). Because the system involves perfusion of only small segments of the jejunum, luminal perfusion with different nutrients did not alter systemic concentrations of perfused nutrients. Therefore observations made can only be ascribed to luminally perfused nutrients. Using this system, the objective of the work described here was to examine the acute effects of luminal nutrients on intestinal mucosal protein metabolism.

Intestinal mucosa is routinely presented with nutrients such as amino acids, glucose, and lipids. Some of these, especially amino acids and glucose, have been shown in different feeding trials to stimulate intestinal growth. We therefore hypothesized that luminal amino acids and glucose would increase mucosal Ks. Along with glutamine, glucose, short-chain fatty acids and ketone bodies are the preferred fuel substrates of the small intestinal epithelial cells (Windmueller and Spaeth, 1980). Because protein synthesis is an energy-consuming process, it was further hypothesized that these fuel substrates would stimulate mucosal protein synthesis in over-night fasted animals. Amino acids are known to suppress whole-body protein degradation as well as degradation in specific tissues such as skeletal muscle (Castellino et al., 19787). Therefore, a final hypothesis was that luminal perfusion with amino acids would suppress the expression of m-calpain and of components of the ATP-ubiquitin proteolytic system. In the ATP-ubiquitin proteolytic pathway, after its activation, ubiquitin, a 76-residue polypeptide is covalently attached to protein substrates by one of a family of ubiquitin conjugating enzymes (E2)

with or without the participation of E3 (protein ligases). Ubiquitin conjugation marks the substrate for ATP-dependent proteolysis by the large proteolytic complex, the 26S proteasome (see Attaix et al., 1998 for a recent review). Intracellular ubiquitin level (Hubbard and Cane, 1994) and mRNA levels of components of this proteolytic system and of m-calpain (Samuels et al., 1996) have been shown to vary in parallel with protein loss seen in intestine during fasting.

MATERIALS AND METHODS

Chemicals

L-[2,6-^{3}H] phenylalanine was purchased from Amersham International (Amersham Place, Little Chalfont Bucks UK). Other chemicals were from Sigma Chemical (St. Louis, MO). Tubing for intestinal cannulation was from Fisher Scientific Company, PA.

Animals and Perfusion Procedure

All experiments were performed in accordance with the Canadian Council on Animal Care Guidelines and were authorized by the institutional Animal Policy and Welfare Committee. Six-week old male piglets (Camborough X Canabrid Pig Improvement Company crosses) were obtained through the University of Alberta Health Sciences Laboratory Animal Services. Piglets were weaned at four weeks and maintained on a wheat/oatgroat-soyabean/whey powder starter diet (crude protein 20.5%; digestible energy 3.6 kcal/kg). Animals were fasted overnight prior to experimentation but water was made available at all times.

Intestinal perfusion was done as described previously (Chapter 2). Briefly, under halothane anesthesia, following a midline incision on the abdomen, four 10-cm jejunal segments were cannulated at both ends (inlet at the pyloric end, outlet at the ileal end) with polyethylene tubing. The inlet cannula for the first segment was inserted 15cm from the Ligament of treitz while successive segments were separated by 50 cm of intestine. Cannulated segments were rinsed of digesta remnants with warm phosphate-buffered saline (PBS, 144.6 mM NaCl, 15.9 mM Na_2HPO_4 1.2 mM $NaH_2PO_4.H_2O$, pH 7.4). Intestinal segments were kept moist by spraying with warm PBS and were covered with PBS-soaked gauze and transparent polyethylene nylon to reduce evaporation. Solutions, at 37.5+0.5°C, were perfused at 3 mL / minutes. In all experiments, the four segments within a piglet were independently but simultaneously perfused.

Experiments

Four to six animals were used in each experiment, as indicated in the Results section (see legends to Tables and Figures).

Effects of Different Luminal Nutrients on Intestinal Mucosal Protein Synthesis.

To examine the effects of luminal nutrients on protein synthesis, one of four intestinal segments within a piglet was perfused with one of the following PBS-based solutions: (A) PBS, (B) 30 mM glutamine, (C) 30 mM amino acids + 50 mM glucose perfused for 40 min, (D) 30 mM amino acids + 50 mM glucose perfused for 90 minutes. In all experiments, treatments were fully randomized to the four intestinal segments within an animal to ensure that any effects of segment position were accounted for. All solutions

were made isoosmotic (300 mosmol / L) with D-manitol and were perfused for 90 minutes except as otherwise indicated. The 30 mM amino acid mixture, with or without 50 mM glucose, was chosen to simulate some of the components of a meal, typical of what the intestinal mucosa would be exposed to in the absorptive state (Ferraris et al., 1990; Adibi and Mercer, 1973; Low, 1979). This amino acid mixture contained (mM) aspartate (0.67), serine (2.03), glutamate (2.34), glutamine (1.84), proline (3.15), glycine (3.75), alanine (2), cystine (0.3), tyrosine (0.79), histidine (0.59), arginine (1.29), asparagine (0.73), threonine (1.34), valine (1.66), methionine (0.55), isoleucine (1.14), leucine (2.09), phenylalanine (2), lysine (1.58), and tryptophan (0.19). Solution B was included because glutamine is a preferred fuel of intestine (Windmueller and Spaeth, 1980) and various studies have shown the importance of the amino acid in stimulating intestinal growth and Ks under different pathophysiological conditions (O'Dwyer et al., 1989; Stein et al., 1994).

In experiment 2, we examined whether the effects of amino acids were concentration dependent. Four intestinal segments within a piglet were perfused with PBS, or with the amino acid solution described above diluted 2x (15 mM), 4x (7.5 mM) or 8x (3.75 mM). Perfusion was for 40 minutes because results from experiment 1 showed that the effect of amino acids was already maximal at 40 minutes.

Based on previous work showing the importance of glutamine in intestinal metabolism, experiment 3 was carried out to examine whether glutamine alone could account for the observed effects of the amino acid mixture. To do this, protein synthesis in intestinal segments perfused with 1.8 mM glutamine (corresponding to the concentration of glutamine in the complete amino acid mixture) was compared to that in segments

perfused with PBS, or 30 mM mixture of the complete amino acid solution. A further comparison involved segments perfused with amino acid mixture in which glutamine and its potential metabolites (glutamate, arginine and proline) had been deleted. To keep the concentration of this mixture at 30 mM, concentrations of glycine (6.62 mM), alanine (4.88 mM) and serine (4.90 mM) were increased. These later amino acids are not thought to exert specific effects on intestinal protein metabolism.

In experiment 4, the effects of energy substrates were examined. Four intestinal segments within a piglet were perfused with PBS, 50 mM glucose, 50 mM mixture of short-chain fatty acids (30 mM acetate, 12.5 mM propionate, and 7.5 mM butyrate), or 20 mM β-hydroxy butyrate. These concentrations are within the physiological ranges (Ferraris et al., 1990; Bergman, 1990; Weber et al., 1982).

Except in the first experiment, perfusion lasted 40 minutes. Protein synthesis was then measured by luminal flooding dose technique as described previously (Chapter 2). Briefly, the intestinal segments were emptied and refilled with same test solutions but containing 2 mM L-[2,6-^{3}H] phenylalanine to give a specific activity of 700 dpm / nmol. Perfusion continued for another 15 minutes. Segments were then removed, emptied, flushed with ice-cold saline and rinsed in two changes of cold saline. Mucosa was scraped on an ice-cold surface, frozen in liquid N_2 and stored frozen at –50°C until analyzed. In the experiment where Northern hybridization was done, samples were stored at –80°C.

Sample Processing and Analysis

Approximately 300 mg of mucosa was powdered in liquid N_2 and then homogenized in 3mL of ice-cold 2% perchloric acid. Samples were then centrifuged at 2, 800 x g for 15 minutes. Supernatants were collected and neutralized with half volume saturated potassium citrate and stored frozen until ready for analysis. Pellets were washed four times with 8ml ice-cold perchloric acid, each time with centrifugation at 3,000 x g. Washed pellets were hydrolyzed in 5 mL 6 N HCl at 110°C for 24 h. Excess HCl was dried off under vacuum.

In line with previous observations that catabolism of phenylalanine by intestinal tissue is minimal (Goodwin et al., 1979), we have shown that when tracer phenylalanine is delivered luminally, all administered radioactivity was associated with phenylalanine, indicating that phenylalanine is not metabolized into other compounds by the intestinal mucosa (Chapter 2). As such, determination of phenylalanine specific radioactivities (sr) in mucosal free and protein-bound fractions was done directly without conversion of the tracer to β-phenylethylamine. Phenylalanine concentration was determined by reversed phase high performance liquid chromatography using pre-column derivation with o-phthaldehyde as described previously (Samuels and Baracos, 1995). [^{3}H]-phenylalanine radioactivity was counted with a Beckman LS 5801 Scintillation counter (Beckman Instruments Inc., Mississauga, Ontario Canada). Fractional rate of protein synthesis (Ks), expressed as % per day, was calculated according to McNurlan et al. (1979),

$$Ks = 100\ S_b / S_f\ t$$

where S_b is the specific radioactivity of protein-bound phenylalanine, t is the duration of isotope perfusion, S_f is the intracellular free phenylalanine sr in tissue samples collected terminally.

Northern Hybridization

Total RNA was isolated from mucosal samples with Trizol™ Reagent (Life Technologies, Burlington, Canada) according to the manufacturer's instructions. Fifteen micrograms of total RNA was electrophoresed in 1% agarose-formaldehyde gels containing ethidium bromide. Gels were run at 100 V for 5 h. RNA was checked visually for integrity of 28S and 18S ribosomal RNA. RNA was transferred to nylon membranes (GeneScreen™, NEN, Boston, MA) by capillary transfer and cross linked to membranes under ultraviolet light at 1, 200 mJ (Stratalinker, Stratagene CA).

Membranes were hybridized with a cDNA sequence encoding rat 14 kDa ubiquitin conjugating enzyme. The cDNA fragment was obtained by digestion with restriction endonucleases Kpn 1 and Bam H1 (Life Technologies, Burlington, Ontario, Canada) and purified using Gene Clean kit (Bio / Can Scientific, Vista, CA). Membranes were prehybridized at 65°C for 2 h and then hybridized overnight at 65°C with ^{32}P-labeled cDNA probes prepared by the random-primer method (Random Primers DNA Labeling System, Life Technologies, Burlington, Ontario, Canada). Prehybridization buffer contained polyvinylpyrrolidone-360, 000 (0.2 %), Ficoll 400 000 (0.2 %), Bovine Serum Albumin (0.2 %), Tris / HCl (0.05 M, pH 7.5), NaCl (1 M), sodium pyrophosphate (0.1 %), SDS (1%), and salmon sperm DNA (50 µg / mL). Hybridization solution differed from prehybridization medium in that it contained ^{32}P-labelled cDNA probe. Following

hybridization, membranes were washed four times in 0.2 X SSC and 0.1 % SDS at 65°C for 15 min each. Membranes were autoradiographed for 24-48 h at –70°C with intensifying screens on X-OMAT™-AR film (Kodak, Rochester, NY). Blots were quantified with BioRad Imaging Densitometer. Northern hybridization analysis for ubiquitin, and C8 and C9 subunits of the proteasome, and of m-calpain we carried out as described previously (Samuels et al., 1996). Differences in RNA loading was corrected for by quantifying the 18S RNA band using a Bio-Rad Imaging Densitometer (Bio-Rad, Hercules, CA).

RESULTS

Luminal Nutrients and Mucosal Protein Synthesis

Free phenylalanine sr was similar for all segments regardless of the luminal perfusate applied (PBS, 30 mM amino acids plus 50 mM glucose, 30 mM amino acids, 30 mM glutamine, Fig. 3-1A). The 30 mM amino acid mixture, without 50 mM glucose, suppressed mucosal protein synthesis by 20-25% relative to PBS (Fig. 3-2B, $P < 0.05$). Lower concentrations of the 30 mM amino acid mixture (3.75, 7.5, and 15mM) did not significantly affects mucosal Ks (relative to PBS, Ks were 91 ± 2, 89 ± 2, 97 ± 2%, respectively in segments perfused with 3.75, 7.5 and 15 mM amino acid mixture, $n = 4$, $P > 0.05$).

Specificity of amino acids involved in regulation of mucosal protein synthesis by luminal amino acids

Perfusion experiments were carried out to examine whether the observed effects of the amino acid mixture on mucosal protein synthesis was due to glutamine. The suppressive effects of amino acids appear to be non-specific, at least with respect to glutamine-related amino acids because protein synthesis was suppressed by the 30 mM amino acid mixture, whether or not glutamine and its related amino acids (glutamate, arginine and proline) were present in the medium (Fig. 3-2). Glutamine alone at its concentration in the complete amino acid mixture (1.8 mM) had no effect on protein synthesis relative to saline (Fig. 3-2). We also examined the effects of these nutrient solutions on mucosal intracellular free amino acid concentrations (Table 3-1). Concentrations of most amino acids reflected their levels in the luminal perfusates. The perfusion of glutamine alone at 1.8 mM (Table 3-1) or at 30 mM (see Table 4-1) significantly raised tissue levels of glutamine, glutamate and ornithine, and tended to increase the concentration of citrulline ($P = 0.08$), although the changes seen in mucosal glutamine concentration were much more pronounced with the 30 mM solution. These changes, which are consistent with known intestinal metabolism of glutamine, were also seen, although with a lesser magnitude, in mucosa segments exposed to the full amino acid mixture (Table3-1). Overall, changes in mucosal amino acid concentrations induced by perfusion with the different amino acid solutions did not have any correlation with changes seen in mucosal protein synthesis.

Effects of Energy Substrates

To examine if the various fuels of the intestinal mucosa would regulate protein synthesis, the effects of 50 mM glucose, 50 mM short-chain fatty acids, and 20 mM ketone bodies were studied. The various energy substrates had no effect on mucosal free phenylalanine sr, or Ks (Fig. 3-3).

Expression of m-calpain, and of Components of ATP-Ubiquitin-Proteasome Proteolytic Pathway

Our finding that luminal amino acids suppressed protein synthesis relative to a saline control was surprising. We speculated that this might be associated with parallel changes in proteolysis. Therefore the effects of the complete amino acid mixture (30 mM) on mucosal mRNA levels of m-calpain, and of components of ATP-ubiquitin-proteasome proteolytic pathway were examined. Northern hybridization analyses were done for m-calpain, ubiquitin, 14 kDa ubiquitin conjugating enzyme, known to be involved in ubiquitin conjugation of substrates for proteolysis (Wing and Banville, 1994), and for C8 and C9 subunits of the 20S proteasome, the proteolytic core of 26S proteasome (Attaix et al., 1998).

There were no effects of luminal amino acids on m-calpain mRNA level (10.7 ± 1 and 10.8 ± 1 Arbitrary units, respectively, for PBS and 30 mM amino acid solution, $n = 8$, $P > 0.05$). As previously reported for rat intestine (Samuels et al., 1996) two ubiquitin transcripts of 2.6 and 1.2 kb were detected. Densitometric analysis on both bands showed that intestinal mucosal total ubiquitin mRNA level was decreased 28% by luminal amino acid perfusion ($P<0.05$, Fig. 3-4A). Luminal nutrient perfusion also significantly

decreased the expression of 1.2 kb transcript of 14 kDa ubiquitin conjugating enzyme by 20% (Fig. 3-4B, $P<0.05$). In parallel to mRNA levels of ubiquitin and 14 kDa E2, expression of C9 subunit of the proteasome was also decreased by 30% by luminal amino acids (Fig. 3-4C, $P<0.05$). There were no effects of luminal treatments on mRNA levels of C8 subunit of the 20S proteasome (6.5 ± 1 and 7.8 ± 1 Arbitrary units, respectively for PBS and amino acid solution, $n = 8$, $P > 0.05$).

DISCUSSION

Our objective was to examine the effects of a variety of nutrients in the first pass on intestinal protein metabolism. Our results suggest a rapid regulation of intestinal protein synthesis and expression of ATP-ubiquitin-proteasome dependent proteolytic system after luminal exposure to amino–acid containing solutions.

Luminal amino acids suppressed intestinal mucosal protein synthesis. The effect of amino acids was seen when intestinal segments were perfused with a 30 mM complete amino acid mixture, or 30 mM amino acid mixture lacking glutamine-related amino acids, or with 30 mM glutamine alone. However the suppressive effects of amino acids on Ks were not seen at lower concentrations of either glutamine alone or of the total amino acid mixture. Although 30 mM amino acid mixture reduced intracellular free phenylalanine level (Table 1), as to be expected from competition for transport by the presence of other amino acids (Hu and Borchardt, 1994; Munck and Munck, 1994), this is unlikely to explain the effects of luminal amino acids because free phenylalanine specific radioactivity, the surrogate pool for the $tRNA_{phe}$ pool, was identical in all treatments.

The suppressive effects of luminal amino acids on intestinal protein synthesis has not been reported before and is contrary to what one might expect based on feeding trials showing stimulatory effects of proteins and amino acids on intestinal protein synthesis and protein mass. The only study similar to this work was that of Weber et al. (1989). These authors luminally perfused a 10-cm segment of jejunum in the rat for about 2 h with 56 mM glucose and showed that glucose perfusion increased mucosal protein synthesis by 20-37%. However, plasma glucose and hormone levels were not measured in that study. Perfusion of a 10-cm segment of the jejunum in rats of the size they used would expose approximately 30% of the jejunum to luminal glucose. Such a situation would most likely increase plasma glucose, glucagon-like peptide 1 (GLP-1), and insulin concentrations. This confounds comparison with the results presented here, in which we have previously demonstrated that there were no systemic effects (Chapter 2). Although the effects of glucagon-like peptides and insulin on intestinal protein synthesis is not yet clear, a recent study has demonstrated the stimulatory effects of IGF-1 on protein synthesis in both mucosal and serosal layers of small intestine of parenterally-fed rats (Lo and Ney, 1996).

There are at least two possible hypotheses to explain the ability of luminal amino acids to suppress mucosal protein synthesis. First, a consideration of intestinal protein metabolism must also include the process of proteolysis, and the tissue could maintain positive protein-balance by a parallel (but greater) fall in proteolytic rate. Although there are no direct methods for estimating intestinal proteolysis, an indirect method involves measurement of mRNA of the different proteolytic systems. The three well characterized intracellular proteolytic systems, ATP-ubiquitin-proteasome, lysosomal and Ca^{2+}-

dependent proteolytic pathways are present in the intestine (Samuels et al., 1996; Hubbard and Carne, 1994; Kishibuchi et al., 1995). Although the ATP-ubiquitin-proteasome pathway is thought to be responsible for much of intracellular proteolysis in skeletal muscle (Attaix et al., 1998), the significance of any of the proteolytic pathways in intestinal proteolysis is not known. In recent studies with fasted rats, ubiquitin level (Hubbard and Carne, 1994) and mRNA levels of m-calpain, and of components of ATP-ubiquitin-proteasome proteolytic pathway (Samuels et al., 1996) varied in parallel with protein catabolism seen in small intestine. We therefore examined the effects of the 30 mM amino acid mixture on protease gene expression. The 30 mM amino acid mixture had no effects on m-calpain expression but suppressed intestinal mucosal expression of ubiquitin, 14 kDa E2, and C9 proteasome subunit in a coordinated manner. No changes in the expression of C8 proteasome subunit were observed. Because the significance of the different steps in regulating the ATP-ubiquitin-proteasome pathway is not known (Attaix et al., 1998; Coux et al., 1996), we can not comment on the implication of lack of changes in mRNA levels of C8 in the presence of changes in the expression of the other components of the pathway measured. In spite of this, our observation of coordinated regulation of ubiquitin, 14 kDa E2, and C9 expression likely indicates a regulation of the ATP-ubiquitin-proteasome pathway by luminal nutrients in intestinal mucosa.

With the model used in this study, it has been shown that perfusion of intestinal segments with different nutrients had minimal or no effects on plasma glucose, amino acid and insulin concentrations (Chapter 2). However in the absorptive state, there are elevated concentrations of absorbed nutrients as well as anabolic hormones and growth factors such as insulin, IGF-1, growth hormone, GLP-1 and –2. Therefore, it is also possible that

the anabolic effect of luminal nutrients is dependent on elevated levels of such growth factors. A recent study with small-bowel resected rats showed that whereas dietary glutamine or IGF-1 infusion alone had no effect on ileal protein content, combined treatments of glutamine and IGF-1 synergistically increased ileal protein content by 20-28% (Zeigler et al., 1996).

The effects of 30 mM luminal amino acids on mucosal protein synthesis could not be attributed to any specific amino acid. Because of the importance of glutamine in regulating intestinal metabolism (O'Dwyer et al., 1989; Stein et al., 1994) we had hypothesized that this amino acid and / or its metabolites (glutamate, arginine, proline, ornithine, citrulline) might be in involved in the observed luminal amino acid-induced suppression of mucosal protein synthesis. However, removal of glutamate, glutamine, arginine and proline from the amino acid mixture did not abolish the effects of the amino acid mixture. Also, glutamine alone at its concentration in the 30 mM amino acid mixture was ineffective in reproducing the effects of the complete amino acid mixture. Although luminal perfusion of glutamine alone at 1.8 mM or 30 mM induced distinctive changes in intracellular amino acid concentrations, changes that are consistent with known intestinal metabolism of this amino acid (Windmueller and Spaeth 1980; Wu et al., 1994), these changes were not necessary for the suppressive effects of amino acid perfusion on protein synthesis to be seen.

A feature of intestinal amino acid metabolism is the production of ammonia, whose concentration in the portal blood can account for up to 18% of amino acid nitrogen intake (Stoll et al., 1998, van Berlo et al., 1988). It is thus possible that ammonia produced in the absorptive state serves as a signal involved in amino acid-induced regulation of

mucosal protein metabolism. Physiological concentrations of NH_4Cl induce cell hypertrophy in cultured renal epithelial cells by mechanisms involving alkalinization of the lysosome and reduced protein degradation (Franch and Preisig, 1996; Ling et al., 1996). It remains to be seen whether ammonia is also used by intestinal epithelial cells as a signaling molecule in the regulation of protein metabolism.

The molecular mechanisms involved in nutritional regulation of intestinal protein synthesis have received little attention. In skeletal muscle, liver, lung and brain, it has been shown that changes in protein synthesis with age and under different nutritional and pathological states can be attributed to changes in peptide–chain initiation step of the translational process (Jurasinski et al., 1995; Kimball, et al., 1992; Vary et al., 1996). Specifically, increases in protein synthesis under some of these conditions were associated with increases in the amount and activity of eukaryotic initiation factors 2 and 2B. No such studies have been carried out in the intestine. However, Samuels et al. (1996) and McNurlan et al. (1979) reported that reduction in intestinal protein synthesis seen 2 or 5 days after fasting correlated with a decrease in ribosomal capacity (RNA / mg tissue protein) but not in translational efficiency (total protein synthesized / tissue RNA). Short term fasting (10-18 h) on the other hand suppressed rat intestinal protein synthesis mainly by reducing efficiency of translation (Burrin et al., 1991). Because of the acute nature of the experiments described in our work, it may be speculated that the alterations seen in protein synthesis were due to changes in translational efficiency rather than changes in ribosomal capacity. Further studies will be needed to examine molecular mechanisms involved in nutritional regulation of intestinal protein synthesis.

Changes in proteolytic gene expression observed in this study were quite rapid, being noticed after 1 h. Other workers have also shown rapid regulation of intestinal epithelial cell expression of ornithine decarboxylase mRNA in response to glutamine, and of intestinal explants expression of sucrase-isomaltase mRNA in response to insulin after 0.3 and 5 h of incubation, respectively (Kandil et al., 1995; Takenoshita et al., 1998). However in the study of Kandil et al (1995), the cells were starved of serum for 4 h prior to the experiments, a treatment that would make the cells quiescent, and therefore represent a high degree of challenge to the cells. The rapid decrease in proteolytic mRNA (within 1.25 h) seen in this study appears to be one of the most rapid nutrient-induced changes in intestinal gene expression.

We did not observe any effects of the energy substrates studied. The piglets used in this study were fasted for only about 16 h, therefore it is possible that the reason we did not see any effects was because the intestine was not sufficiently starved. Glucose may also promote protein anabolism through suppression of proteolysis (Tessari et al., 1996), an aspect that was not examined in this study.

Taken together, these data on protein synthesis and mRNA measurements might imply that luminal nutrients regulate intestinal protein metabolism in an energy efficient manner. Protein synthesis and degradation are energetically expensive (Goldberg et al., 1980; Newsholme and Leech, 1985). For instance, the formation of a single peptide bond at the translational stage alone involves the consumption of at least 5 moles of ATP (Newsholme and Leech, 1985). Therefore the synthesis of a polypeptide such ornithine decarboxylase, with 461 amino acid residues (Yao et al., 1995), will involve the utilization of considerable amount of energy. Although the details of the energetic of

ATP-ubiquitin-proteasome proteolytic system is yet to be worked out, activation of each ubiquitin molecule requires 1 mole of ATP. Since about 5-8 ubiquitin molecules are typically conjugated to one protein substrate (Mitch and Goldberg, 1996) an energy equivalent of more than 20g of glucose is consumed for ubiquitin activation alone. This is in addition to ATP requirements for the assembly of the 26S proteasome, the unfolding of the protein substrates and possibly their injection into the proteolytic chamber of the 26S proteasome, and the breakdown of ubiquitinated proteins into peptides (Attaix et al., 1998). Because of these energetic costs of protein synthesis and degradation, it appears to be more efficient for the intestine to achieve positive N balance by suppressing both processes than by increasing protein synthesis. This strategy may also enable the intestine to be in positive N balance during the absorptive state while still delivering amino acids to the general circulation to be presented to all tissues on an as-needed basis.

Table 3-1. Luminal Nutrients Perfusion and Mucosal Free Amino Acid Concentrations[a]

[a]Values are means of 6 piglets. [b]Pooled standard Error of means. [cde]Means with different superscripts within a row are significantly different from one another ($P < 0.05$, $n = 6$). One of four intestinal segments within the same piglet was perfused with phosphate-buffered saline (PBS, pH 7.4) or 30 mM amino acid mixture lacking glutamine, arginine, glutamate, and proline (30 mM AA-no Gln); or 1.84 mM glutamine, or 30 mM complete amino acid mixture (30 mM AA) for a total of 55 minutes.

	PBS	30 mM AA - no Gln	1.84 mM Gln	30 mM AA	SE[b]
Essential AA					
Methionine	106[c]	225[d]	118[c]	265[d]	46
Phenylalanine	845[c]	521[d]	987[c]	534[d]	82
Valine	252[c]	942[d]	294[c]	962[d]	137
Isoleucine	146[c]	536[d]	173[c]	561[d]	82
Leucine	134[c]	595[d]	148[c]	605[d]	90
Threonine	191[c]	639[d]	217[c]	535[d]	82
Lysine	136	156	92	166	38
Non Essential AA					
Aspartate	871[c]	876[c]	1618[d]	1517[d]	181
Glutamate	3123[cd]	2811[c]	4828[e]	4313[de]	411
Asparagine	69[c]	545[d]	75[c]	521[d]	66
Serine	374[c]	5367[d]	365[c]	2189[e]	389
Glutamine	427[c]	389[c]	1800[d]	817[c]	162
Glycine	817[c]	1888[d]	837[c]	1570[d]	154
Citrulline	248	329	402	416	56
Arginine	108[c]	97[c]	142[c]	593[d]	106
Alanine	701[c]	3836[d]	1555[ce]	2320[e]	355
Tyrosine	86[c]	366[d]	108[c]	568[d]	54
Ornithine	22[cd]	17[c]	26[c]	25[d]	2

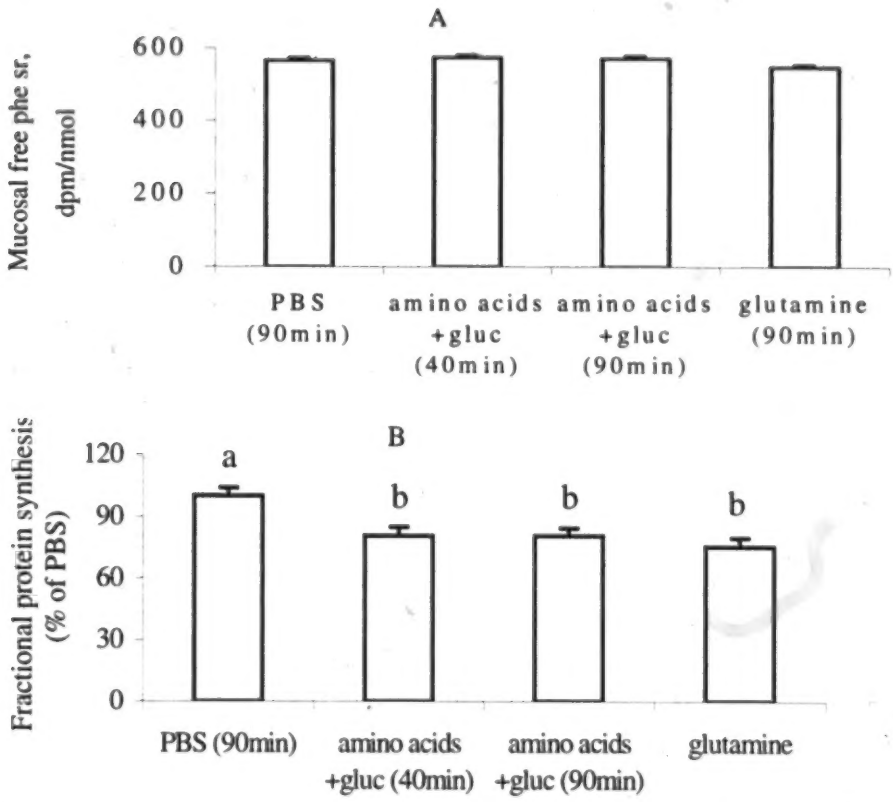


Figure 3-1. Mucosal Free Phenylalanine Specific Radioactivity (A, dpm / nmol) and Ks (B, expressed as % of Control (PBS) Solution) in Piglet Jejunal Segments.

Intestinal segments were luminally perfused with one of four solutions: phosphate buffered saline (PBS, pH 7.4); 30 mM amino acid mixture + 50 mM glucose (Amino acids + gluc) perfused for 40 or 90 minutes; 30 mM glutamine in PBS (Glutamine). Mucosal protein synthesis in perfused segments was then measured as described in Materials and Methods. Ks in control (PBS) segment was 42 ± 2 % / day. Values are means ± SE (standard error of the means) for n =6. Bars with different superscripts are significantly different ($P < 0.05$).

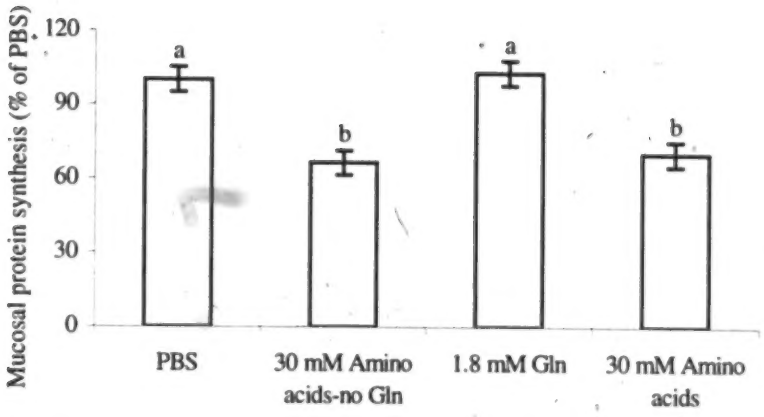


Figure 3-2. Effects of Luminal Nutrient Solutions on Mucosal Fractional Protein Synthetic Rates.

Piglet jejunal segments luminally perfused with one of four solutions: Phosphate buffered saline, (PBS, pH 7.4); 30 mM amino acid mixture lacking glutamine, arginine, glutamate, and proline (30 mM amino acids – no glutamine); 1.84 mM glutamine (the concentration of the amino acid in the complete amino acid mixture); 30 mM amino acid mixture. After perfusing the stated solutions for 40 minutes, protein synthesis was measured as described in legend to Fig. 1 and in Materials and Methods. Ks in control (PBS) segment was 51 ± 4 %. Values are means ± SE for n = 6. Bars with different superscripts are significantly different ($P < 0.05$).

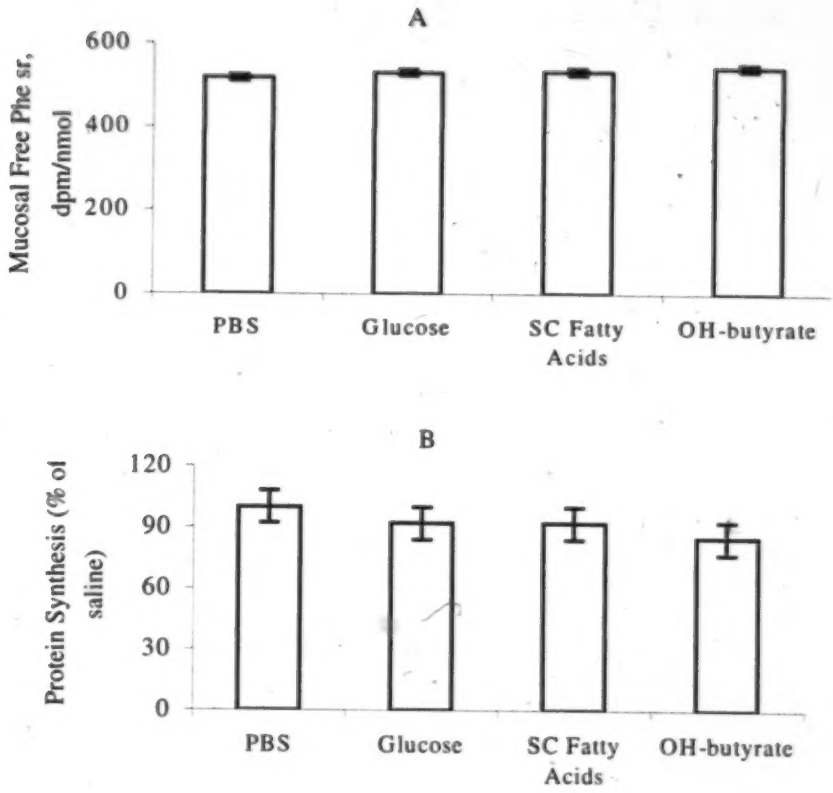
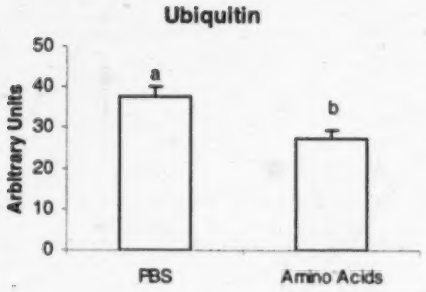


Figure 3-3. Effects of Energy Substrates on Mucosal Free Phenylalanine Specific Radioactivity (A, dpm / nmol) and Ks (B, expressed as % of control (PBS) solution). Piglet jejunal segments were luminally perfused with PBS (phosphate buffered saline, pH 7.4) or glucose (50 mM) or short-chain fatty acids (50 mM) or β-hydroxy butyrate (20 mM). After perfusion for 40 minutes, protein synthesis was measured as described in legend to Fig. 1 and in Materials and Methods. Ks in control (PBS) segment was 39 ± 2 % / day. Values are means ± SE for n = 4. Bars with different superscripts are significantly different ($P > 0.05$).

Figure 3-4. Effects of Luminal Nutrients on mRNA Levels of Components of ATP-Ubiquitin-Proteasome Proteolytic Pathway.

One of two intestinal segments within the same piglet was perfused with PBS or 30 mM mixture of the 20 amino acids as described in Material and Methods. Total mucosal RNA was separated on agarose gel, transferred to nylon membrane and hybridized with ^{32}P-labeled cDNA encoding ubiquitin, 14 kDa E2, and C9 subunit of 20S proteasome as described in Material and Method. Data refer to densitometric signals of all transcripts (ubiquitin) and to 1.2-kb transcript (14-kDa E2). Values are means ± SE for n = 8. Bars with different superscripts are significantly different ($P < 0.05$). Representative Northern blots are shown for 14 kDa E2.


Ubiquitin
Arbitrary Units
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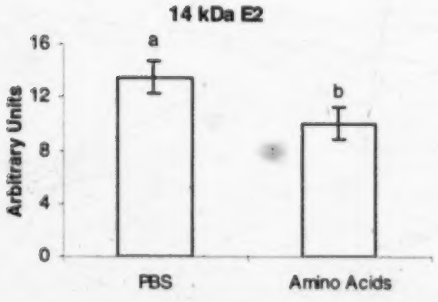
40
30
20
10
0
a
b
PBS
Amino Acids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Arbitrary Units
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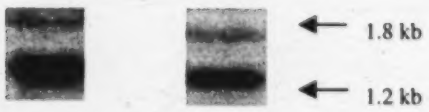
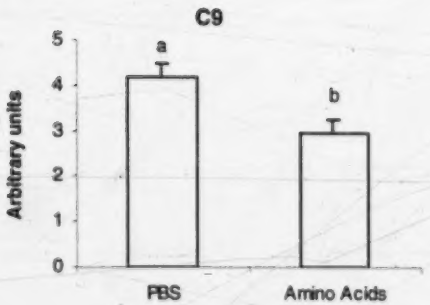
12
8
4
0
a
b
PBS
Amino Acids


1.8 kb
1.2 kb


C9
Arbitrary units
5
4
3
2
1
0
a
b
PBS
Amino Acids

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CHAPTER 4

Jejunal Mucosal Protein Turnover: Simultaneous Effects of Luminal And Intravenous Nutrients

INTRODUCTION

Protein balance is the result of the relative rates of protein synthesis and protein degradation in all tissues. In the intestine, an additional complexity is that these processes may be responsive to apical and basolateral stimuli. Luminal nutrients could be locally effective in the regulation of intestinal protein synthesis and degradation. Since total parenteral feeding, when compared to oral feeding, leads to intestinal atrophy, and a decrease in tissue protein content and rates of protein synthesis (Burrin et al., 1994; Dudley et al., 1998; Stein et al., 1994), the small intestine is clearly dependent upon a luminal supply of nutrients in order to maintain positive N balance.

We recently described a perfusion system that allows study of the acute effects of luminal nutrients on intestinal protein metabolism (Chapter 2). Because the system involves perfusion of only small segments of the jejunum, luminal perfusion did not alter systemic concentrations of perfused nutrients or insulin. Therefore modifications in protein metabolism can be ascribed to luminally perfused nutrients. Using the system, we showed that luminal perfusion of intestinal segments with a 30 mM mixture of amino acids suppressed protein synthesis by 20-25% relative to a control saline solution but the energy substrates did not have any effect (Chapter 3). While there are presently no direct methods for measuring intestinal proteolysis *in vivo*, the mRNA levels of components of

the different proteolytic systems have been used as an index of protein catabolism in a variety of tissues (Baracos et al., 1995; Samuels et al., 1996). Luminal perfusion with an amino acid mixture induced a rapid reduction in the levels of mRNA encoding various components of the ATP-ubiquitin-proteasome proteolytic system (Chapter 3).

The results of these studies suggest a coordinated reduction in synthesis and degradation of protein after acute exposure to luminal amino acids, a response, which would maximize transit of absorbed amino acids into the body and minimize their retention within the tissue. These results, however, were unexpected, based on numerous studies showing increased intestinal protein accretion and synthesis after feeding (Davis et al., 1996; McNurlan et al., 1979; McNurlan and Garlick, 1981; Wykes et al., 1996). Net anabolism associated with elevated rates of protein synthesis would be expensive both in terms of energetic cost as well as substrates. This anabolism would seem justified only in circumstances of nutritional adequacy / plenty and would presumably be signaled either by the presence of absorbed nutrients systemically or by hormones released in the absorptive state. We therefore hypothesized that regulation of intestinal protein metabolism is also dependent on systemic signals reflecting overall nutritive status of the animals and that such signals would permit elevations of protein synthesis as seen after feeding *in vivo*. A principal candidate for systemic regulation would be insulin, which promotes protein anabolism in many tissues in response to feeding (Davies et al., 1996; Gelfand and Barret, 1987; Moldawer et al., 1980; Tessari et al., 1996; Wray-Cahen et al., 1997). It is also conceivable, however, that net anabolism may be provoked by the simultaneous presence of luminal nutrients and systemic anabolic signals.

This work examined the acute effects of luminal nutrients on intestinal mucosal protein metabolism under a condition when the overall nutritive status of the animal was either maintained at a fasting level or increased by intravenous infusion of glucose which is accompanied by elevated plasma glucose and insulin concentrations.

MATERIALS AND METHOD

Chemicals

Sterile 75% dextrose and saline were obtained from the University of Alberta Hospital. L-[2,6-^{3}H] phenylalanine was purchased from Amersham International (Amersham Place, Little Chalfont Bucks UK). Other chemicals were from Sigma Chemical (St. Louis, MO).

Animal, Surgery and Perfusion Procedure

All experiments were performed in accordance with the Canadian Council on Animal Care Guidelines and were authorized by the institutional Animal Policy and Welfare Committee. Six-week old male piglets, weighing 12-15 kg, (Camborough X Canabrid Pig Improvement Company crosses) were obtained through the University of Alberta Health Sciences Laboratory Animal Services. Piglets were weaned at four weeks and maintained on a wheat / oatgroat-soyabean / whey powder starter diet (crude protein 20.46%; digestible energy 3.55 kcal/kg). Animals were fasted overnight but water was made available at all times.

Anesthesia was induced with a mixture of Torbugesic (0.2 mg/kg), Ketamine (11 mg/kg), Rompun (2.2 mg/kg) and glycopyrrolate (0.01 mg/kg), and maintained with 2%

halothane delivered with oxygen. Catheters were placed in the right and left jugular veins for infusion and blood sampling, respectively. After the placement of catheters, lines were filled with heparinized saline while intestinal cannulation was done (see below). After the commencement of intestinal perfusion, piglets (n =6) were injected with 5 mL of 50 % dextrose through the infusion catheter and thereafter infused the dextrose solution at the rate of 20 mg/ kg/ h to the end when intestinal segments were taken out after luminal isotope perfusion (see below). Another 6 piglets were infused with saline. Two piglets were studied each day, one infused with glucose and the other with saline, with saline infusion rate being fixed at the rate of dextrose infusion. The rate of dextrose infusion was chosen based on review of the literature on similar studies and after a preliminary experiment showing a plateau in glucose and insulin concentrations between forty to sixty minutes of the start of infusion in dextrose infused piglets (see Appendix 3). Insertion of intestinal cannula and luminal perfusion of intestinal segments were done as described previously (Chapter 2). Briefly, following a midline incision on the abdomen, four 10-cm length jejunal segments were cannulated at both ends (inlet at the pyloric end, outlet at the ileal end) with polyethylene tubing. Inlet cannula for the first loop was inserted 15cm from the ligament of treitz while successive loops were separated by 50 cm of intestine. Loops were rinsed of digesta remnants with warm phosphate-buffered saline (PBS, 144.6 mM NaCl, 15.9 mM Na_2HPO_4 1.2 mM $NaH_2PO_4.H_2O$, pH 7.4. Solutions, at 37.5+0.5°C, were perfused at 3 mL / minutes. In all experiments, the four segments within a piglet were independently but simultaneously perfused. Within each intravenous infusion group, four intestinal segments within the same piglets were perfused with one of the following PBS-based solutions: (i) PBS, or (ii) 30 mM amino acid mixture + 50

mM glucose, or (iii) 30 mM amino acid mixture, or (iv) 30 mM glutamine. All solutions were made isoosmotic (300 mosmol / L) with D-manitol and perfused for 60 minutes. Luminal treatments were fully randomized to the four intestinal segments within an animal to ensure that any effects of segment position were accounted for. Solutions (ii) and (iii) were chosen to simulate some of the components of a meal, typical of what intestinal mucosa would be exposed to in the absorptive state (Adibi and Mercer, 1973; Low, 1979; Ferraris et al., 1990). The amino acid mixture contained (mM) aspartate (0.67), serine (2.03), glutamate (2.34), glutamine (1.84), proline (3.15), glycine (3.75), alanine (2), cystine (0.3), tyrosine (0.79), histidine (0.59), arginine (1.29), asparagine (0.73), threonine (1.34), valine (1.66), methionine (0.55), isoleucine (1.14), leucine (2.09), phenylalanine (2), lysine (1.58), and tryptophan (0.19). Solution (iv) is included because glutamine is the preferred fuel of intestinal epithelial cells (Windmueller and Spaeth, 1980). These three nutrient solutions were shown in our previous study to suppress intestinal mucosal protein synthesis by 20 to 25% (Chapter 3).

After perfusion for 1 h, protein synthesis was measured by luminal flooding dose technique as described Chapter 2. Briefly, the intestinal segments were emptied and refilled with same test solutions but containing 2 mM L-[2,6-^{3}H] phenylalanine to give a specific activity of 700 dpm / nmol. Perfusion continued for another 15 minutes. Segments were then removed, emptied, flushed with ice-cold saline and rinsed in two changes of cold saline. Mucosa was scraped on an ice-cold surface, frozen in liquid N_2 and stored frozen at –80°C until analyzed. Plasma from blood samples taken pre-surgery and at 15-minute's interval from the start of perfusion were stored frozen at –20°C.

Sample Processing and Analysis

Determination of fractional rates of protein synthesis (Ks) in mucosal samples were done according to (McNurlan et al., 1979), with modifications for direct determination of phenylalanine specific radioactivity as described previously (Chapter 2).

Plasma glucose concentration was measured using Glucose (Trinider) Kits (Sigma Diagnostics, St. Louis, MO) while plasma insulin concentration was determined using Enzymun-T Insulin Assay (Boehringer Mannheim Immunodiagnostics, Laval Quebec, Canada). Amino acid concentrations were determined by high performance liquid chromatography (Strelkov et al., 1989).

Northern Hybridization

Mucosal total RNA was isolated with Trizol™ Reagent (Life Technologies, Burlington, Canada) according to the manufacturer's instructions. Fifteen micrograms of total RNA was electrophoresed in 1% agarose-formaldehyde gels containing ethidium bromide. Gels were run at 100 V for 5 h. RNA was checked visually for integrity of 28S and 18S ribosomal RNA. RNA was transferred to nylon membranes (GeneScreen™, NEN, Boston, MA) by capillary transfer and cross linked to membranes under ultraviolet light at 1, 200 mJ (Stratalinker, Stratagene CA). Differences in RNA loading was corrected for by quantifying the 18S RNA band using a Bio-Rad Imaging Densitometer (Bio-Rad, Hercules, CA)

Membranes were hybridized with a cDNA sequence encoding rat 14 kDa ubiquitin conjugating enzyme (E2). The cDNA fragment was obtained by digestion with restriction

endonucleases Kpn 1 and Bam H1 (Life Technologies, Burlington, Ontario, Canada) and purified using Gene Clean kit (Bio / Can Scientific, Vista, CA). Membranes were prehybridized at 65°C for 2 h and then hybridized overnight at 65°C with ^{32}P-labeled cDNA probes prepared by the random-primer method (Random Primers DNA Labeling System, Life Technologies, Burlington, Ontario, Canada). Prehybridization buffer contained polyvinylpyrrolidone-360, 000 (0.2 %), Ficoll 400 000 (0.2 %), Bovine Serum Albumin (0.2 %), Tris / HCl (0.05 M, pH 7.5), NaCl (1 M), sodium pyrophosphate (0.1 %), SDS (1%), and salmon sperm DNA (50 µg / mL). Hybridization solution differed from prehybridization medium in that it contained ^{32}P-labelled cDNA probe. Following hybridization, membranes were washed four times in 0.2 X SSC and 0.1 % SDS at 65°C for 15 min each. Membranes were autoradiographed for 24-48 h at –70°C with intensifying screens on X-OMAT™-AR film (Kodak, Rochester, NY). Blots were quantified with BioRad Imaging Densitometer. Northern hybridization analysis for ubiquitin, and C8 and C9 subunits of the proteasome we carried out as described previously (Samuels et al., 1996).

Data are expressed as means ± SE (standard error of the means) and were analyzed by analysis of variance. Differences amongst means were examined using Fishers Protected Least Significant Difference using SAS statistical package (1982) and were considered significant if $P < 0.05$.

RESULTS

Pre-infusion plasma glucose and insulin levels did not differ significantly in the glucose- and saline-infused piglets (Glucose: 6.0 ± 0.9 vs 5.0 ± 0.3 mM; Insulin: 8.5 ± 0.7 vs 6.5 ±

1.14 μU / mL, for glucose- and saline-infused piglets, respectively) but were increased by 300% in glucose-infused piglets (Fig. 4-1). However, whereas the increase in glucose was rapid and remained constant in the last 30 minutes of the study, the increase in insulin did not occur until the last 15 minutes of infusion (Fig. 4-1A). Plasma insulin concentration at this time (20 ± 2 μU) was lower than that observed in fed 4-week old piglets (36 μU, Davis et al., 1993). Intravenous glucose infusion increased mucosal protein synthesis by 16% ($P < 0.05$, $n = 6$), (Fig. 4-2). Because there were no interactions, data on the effects of luminal nutrients were pooled. The different luminal nutrient solutions (30 mM amino acids with or without 50 mM glucose, or 30 mM glutamine) suppressed mucosal protein synthesis by 10% relative to segments perfused with PBS ($P < 0.05$, n=12, Fig. 4-2).

We were also interested in examining the effects of the various treatments on intestinal proteolysis. We did this indirectly by examining the mRNA levels of components of ATP-ubiquitin-proteasome proteolytic systems: ubiquitin, 14 kDa ubiquitin conjugating enzyme (E2), and of C8 and C9 components of the proteasome. In this pathway, activated ubiquitin is covalently attached to protein substrates by one of a family of ubiquitin conjugating enzymes (E2) with or without the participation of E3 (protein ligases). Ubiquitin conjugation marks the substrate for ATP-dependent proteolysis by the large proteolytic complex, the 26S proteasome (see Attaix et al., 1998 for a recent review). There were no effects of intravenous treatments on the expressions of these components, therefore data for luminal nutrients have been pooled. Except for the solution 30 mM amino acids plus 50 mM glucose, the various luminal nutrients (30 mM amino acids or 30 mM glutamine) suppressed the expression of ubiquitin, 14 kDa

ubiquitin conjugating enzyme, and proteasome subunit C9 by 30% relative to PBS (Fig. 4-3 to 4-5). There were no effects of any treatments on proteasome subunit C8 expression (see Appendix 4). Finally, we also examined the effects of the various treatments on mRNA levels of Ca^{2+}-dependent proteolytic system. mRNA levels of mcalpain, one of the two calpains that differ in their affinity for calcium, were not affected by luminal or intravenous treatments (Fig. 4-7).

Plasma and Mucosal Free Amino Acid Concentrations

To gain further insights into the intravenous and luminal treatments-induced changes in protein synthesis and protease gene expression, we examined the effects of these treatments on plasma and mucosal intracellular free amino acids. The only effect of intravenous glucose infusion seen was in the concentration of alanine, whose level was slightly but significantly lower in the saline-infused animal compared to the level in glucose-infused piglets (1282 ± 92 and 1008 ± 84 μmol / mL, respectively in glucose- and saline-infused piglets, $P<0.05$).

Free mucosal ornithine concentration was significantly increased (3.9 ± 0.1 vs 3.4 ± 0.1 nmol / g wet mucosa, $P = 0.03$, $n = 4$), and that of arginine tended to be higher (298 ± 13 vs 245 ± 13 nmol / g wet mucosa, $P = 0.06$) in the glucose-infused piglets. The effects of luminal treatments on intracellular free amino acids (Table 4-1) generally followed three patterns: 1) Concentrations of most amino acids reflected their concentrations in the perfusates, 2) The presence of 50 mM glucose combined with 30 mM amino acids in perfusates significantly reduced intracellular levels of most amino acids when compared with perfusion of 30 mM amino acids alone, 3) Glutamine perfusion alone increased

concentrations of aspartate, glutamate, arginine, alanine, and ornithine relative to PBS perfusion.

DISCUSSION

We have demonstrated that basolateral, rather than luminal, stimulus was effective in stimulating mucosal protein synthesis. The basolateral stimulus provided (glucose) increased mucosal protein synthesis by 16%. This increase is similar to a value of about 18% seen after re-feeding in fasted piglets (Davis et al., 1996), demonstrating that glucose (and or the associated increase in plasma insulin) could account for feeding-induced increase in mucosal protein synthesis. Findings in this study also confirmed and extended earlier observations made in this laboratory on the potency of luminal nutrients, when provided alone in the intestine, to acutely regulate intestinal mucosal protein metabolism.

We conjectured that intestinal protein turnover would be sensitive to stimuli coming from both apical and basolateral sides. A number of apical stimuli, including amino acids (with or without glucose) and glutamine, suppressed protein synthesis. None of the factors applied apically, including glucose (and other energy substrates, see Chapter 3), amino acids and glutamine, activated protein synthesis, but the effects of the only basolateral stimulus applied (glucose infusion) was the contrary, with the stimulus increasing protein synthesis. Ks was consistently higher in intestinal segments from glucose infused animals as compared to corresponding segments in saline-infused piglets, i.e., in animals receiving only luminal nutrients. This points to a clear sidedness in intestinal response to protein synthesis. By contrast, the basolateral stimulus was without effects on the

expression of proteolytic genes as only the luminal amino acids had suppressive effects on this parameter. It remains to be seen whether the luminal treatments which suppressed the expression of protease genes also have the capacity to increase protein synthesis and / or suppress protein degradation if applied intravenously.

We do not know of any previous study to which this work can be compared. Some studies have shown that glucose and insulin regulate muscle and whole body protein metabolism at the level of both protein synthesis and degradation (Gelfand et al., 1987; Larbaud et al., 1996; Moldawer et al., 1980; Tessari et al., 1996; Jeevanandam et al., 1991). The stimulatory of effects of intravenous glucose infusion on mucosal protein synthesis observed in this study is consistent with these previous observations. It is however worthy of note that in a previous study (Chapter 3), we could not demonstrate any stimulatory effects of luminally perfused glucose, possibly indicating that to receive signals for regulation of mucosal protein synthesis, the intestinal mucosal tissue discriminates between its two membranes (luminal or basolateral).

In addition to elevated plasma glucose level in the glucose-infused piglets, we also observed elevated, albeit delayed, level of insulin these piglets. Therefore the increase in protein synthesis observed could be due to the effects of glucose, insulin, or both. In a recent study with 7 and 26-d old fasted piglets, re-feeding-induced increase in jejunal protein synthesis was associated with elevated plasma insulin level (Davis et al., 1996). A more recent study from the same laboratory however showed that hyperinsulinemic (euglycemic- amino acid clamps) had no effects on intestinal protein synthesis in fasted 7-d old piglets (Davis et al., 1998). This finding might indicate that glucose, rather than

insulin, might be the basolateral signal that increased mucosal protein synthesis. It is also possible that the two might be interacting.

The mechanism by which glucose (alone or in combination with insulin) would increase protein synthesis is not clear. Although intravenous glucose treatment increased plasma alanine concentration, this amino acid is not known to regulate intestinal protein metabolism. Intracellular ornithine and arginine concentrations were higher in piglets infused with glucose, indicating that glucose infusion might increase intestinal glutamine utilization (Wu et al., 1995). Because ornithine is a precursor of polyamines, which have been associated with the regulation of protein synthesis and growth of the intestine (Kaouass et al., 1996; Noguchi et al., 1996), this amino acid may be involved in the intravenous glucose treatment-induced increase in protein synthesis. However, ornithine is not known to regulate protein synthesis in any tissue. Alternatively, the elevated glucose may serve as an energy source to fuel protein synthesis.

Some of the studies that have demonstrated the suppressive effects of glucose or insulin on tissues and whole body proteolysis were generally carried out with infusion periods of 4 h or more (Gelfand et al., 1987; Larbaud et al., 1996; Moldawer et al., 1980; Tessari et al., 1996). It is thus possible that the time allowed in this study was not long enough for the effects of glucose / insulin on proteolysis to be seen. Only one study has examined the effect of insulin infusion on the intestine specifically. Larbaud et al. (1996) showed 6 h infusion of the hormone had no effects on the expression of components of ATP-ubiquitin-proteasome, lysosomal, and Ca^{2+}-dependent proteolytic systems in the intestine of adult goats. There is too little information presently available to appreciate the roles of insulin and glucose in the regulation of intestinal protein turnover. A series of

physiological clamp experiments in which insulin and glucose concentrations are systematically and independently varied would great clarify this area.

Luminal treatments

In line with the study described in Chapter 3, luminal nutrients (30 mM mixture of the 20 amino acids with or without 50 mM glucose, and 30 mM glutamine) suppressed mucosal protein synthesis. The expression of ubiquitin, 14 kDa ubiquitin conjugating enzyme, and proteasome subunit C9 were also suppressed by luminal nutrients. This was observed with luminal perfusion of the complete amino acid mixture or glutamine alone. A further observation in this work is that co-perfusion of glucose and amino acids blunted the effects of amino acids on the expression of components of ATP-ubiquitin-proteasome proteolytic systems. The mechanism by which this happens is not clear. However, we observed that the concentrations of total amino acids, of total essential and non-essential amino acids, and of most individual amino acids were significantly higher in intestinal segments perfused with the amino acid mixture alone, compared to segments perfused with a mixture of both amino acids and glucose (Table 4-1).

A possible source of intracellular amino acids is proteolysis. This would not however explain the differences in free intracellular amino acid concentrations as the mRNA data suggest that proteolysis might be higher in the intestinal segments perfused with the solution containing amino acids plus glucose. Rather the differences in intracellular free amino acid concentrations appear to be a transport effect because mucosal intracellular concentrations of citrulline and ornithine, amino acids not included in the perfusates but which the intestine can synthesize (Windmueller, 1982), were not affected. Rerat (1993)

and Rerat et al. (1992) have suggested that the presence of luminal sugars may limit the inward transport of amino acids. It is therefore possible that the lack of effects of amino acids on the expression of genes of components of ATP-ubiquitin-proteasome proteolytic system in intestinal segments perfused with both amino acids and glucose was due to reduced uptake and the resultant reduced free amino acids those segments. However since protein synthesis was similarly suppressed by luminal amino acids with or without glucose, such a regulation will imply that mucosal protein synthesis and degradation (as reflected in expression of proteolytic genes) may be independently regulated.

Samuels et al. (1996) observed a 151% increase in m-calpain mRNA level following one day fasting. Neither intravenous nor luminal treatments exerted any effects on mucosal m-calpain mRNA levels in this study. Whether this was due to the acute nature of the treatments is not known. Further studies will be needed to examine the contributions of the various proteolytic systems to intestinal proteolysis.

In conclusion, intravenous glucose infusion increased mucosal protein synthesis but had no effects on the expression of proteolytic genes. Luminal amino acids (with or without glucose) on the other hand suppressed protein synthesis, clearly showing that intestinal mucosa demonstrated sidedness in its response to protein synthesis signals. It appears that luminal amino acids independently regulate intestinal protein synthesis and expression of genes involved in proteolysis, regardless of changes within normal ranges of systemic glucose and / or insulin concentrations. However, this study also suggests that the direct effects of luminal amino acids on proteolytic genes may be modulated by the presence of glucose within the lumen or first pass transport and / or metabolism of glucose. Finally, it was demonstrated that luminal and basolateral stimuli regulate intestinal protein

metabolism by different mechanisms. Attempts to control intestinal atrophy are of limited efficacy if both protein synthesis and degradation are not controlled. It would appear critical to establish which apical stimuli are most effective in suppressing protein degradation and which basolateral stimuli, if any, have this effect. Understanding of basolateral factors involved in stimulating protein synthesis is equally important.

Table 4-1. Effects of Luminal and Intravenous Treatments on Mucosal Free Amino Acid concentrations[a]

[a]Values are in nmol /g wet mucosa. One of four intestinal segments within a piglet was perfused with phosphate buffered saline (PBS, pH 7.3), or with 30 mM amino acid mixture plus 50 mM glucose, or 30 mM amino acid mixture, or 30 mM glutamine for 1.25 h. Mucosal samples from perfused segments were processed as described under Materials and Methods. [bcde] Values within a row with different superscripts significantly differ from one another ($P < 0.05$).

	PBS	Amino Acids plus glucose	Amino Acids	Glutamine	Pooled SE
Essential AA					
Methionine	28^{b}	107^{c}	160^{d}	35^{b}	6
Tryptophan	13^{b}	22^{c}	29^{d}	17^{b}	1
Phenylalanine	597^{b}	221^{c}	305^{d}	238^{c}	21
Valine	204^{b}	434^{c}	618^{d}	217^{b}	28
Isoleucine	108^{b}	267^{c}	397^{d}	115^{b}	18
Leucine	96^{b}	293^{c}	434^{d}	103^{b}	17
Threonine	235^{b}	288^{b}	367^{b}	207^{b}	57
Lysine	32^{b}	65^{c}	69^{c}	41^{b}	4
Total	1319^{b}	1717^{c}	2405^{d}	952^{b}	126
Non Essential AA					
Aspartate	392^{b}	495^{b}	731^{c}	1136^{d}	54
Glutamate	1849^{b}	2218^{bc}	2512^{c}	4372^{d}	200
Asparagine	78^{b}	332^{c}	558^{d}	108^{b}	28
Serine	185^{b}	842^{c}	1355^{d}	208^{b}	54
Glutamine	310^{b}	809^{b}	569^{b}	10548^{c}	644
Glycine	1884^{b}	3290^{c}	3832^{c}	1528^{b}	227
Citrulline	184^{b}	288^{bc}	394^{c}	279^{bc}	48
Arginine	126^{b}	357^{c}	521^{d}	207^{e}	24
Taurine	3274^{b}	2179^{c}	2235^{c}	1957^{c}	269
Alanine	475^{b}	1114^{c}	1497^{d}	1096^{c}	78
Tyrosine	65^{b}	153^{c}	226^{d}	94^{e}	9
Ornithine	3^{b}	4^{c}	5^{c}	4^{c}	0.3
Total	8824^{b}	12082^{c}	15679^{d}	22273^{e}	1031
Total	10183^{b}	13840^{c}	18317^{d}	23317^{e}	1069

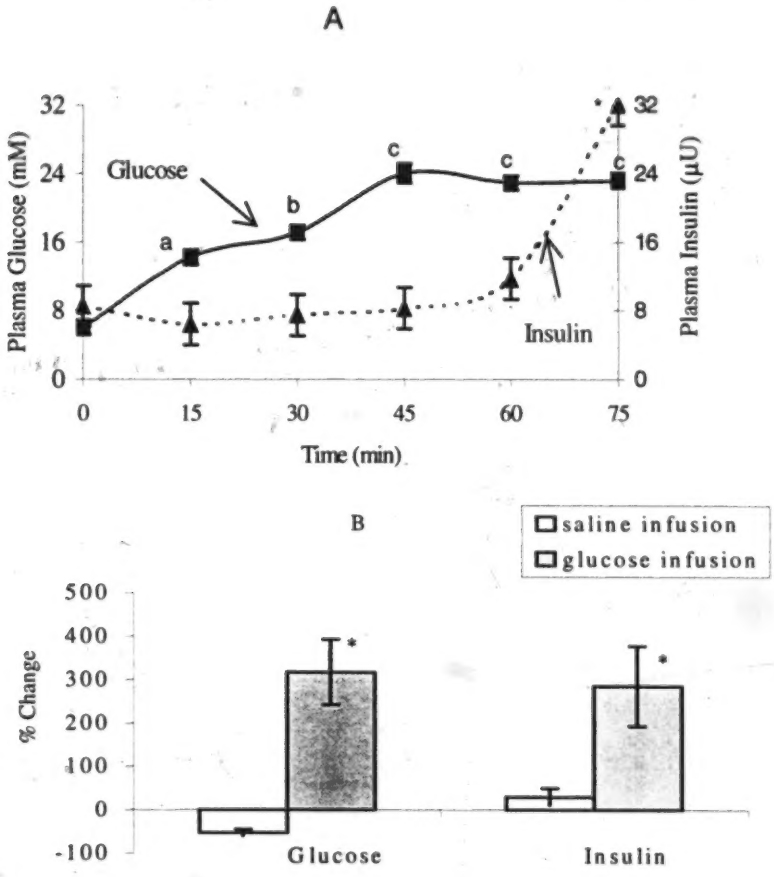

Figure 4-1. Effects of Luminal and Intravenous Treatments on Plasma Glucose and Insulin Concentrations.

Piglets were intravenously infused with glucose (20 mg. Kg^{-1}. h^{-1}, n = 6) or saline for 1.25 h. Blood samples collected before and at 15-minute intervals during infusion were analyzed as described under Materials and Methods. A: Changes in plasma glucose and insulin concentrations as a function of time in glucose-infused piglets. B. Changes in plasma glucose and insulin levels at 75 minutes of infusion, expressed as % change from pre-infusion values.

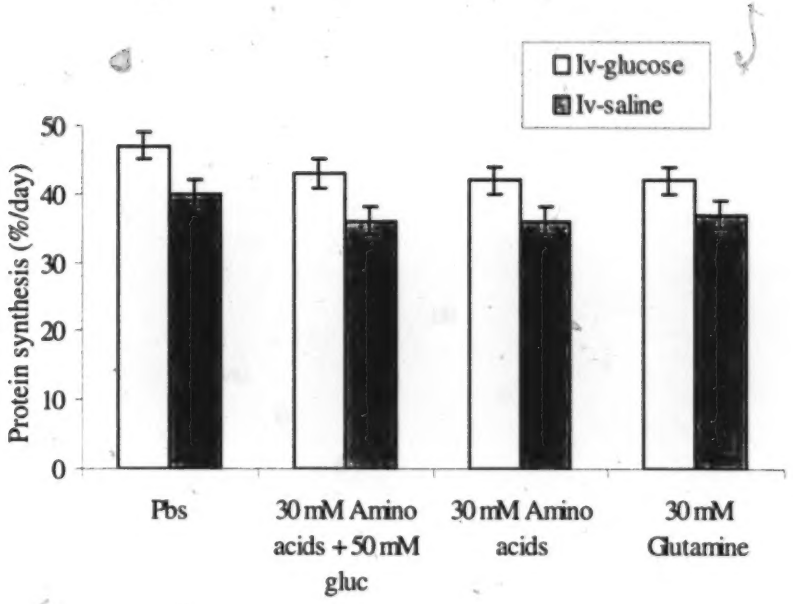

Figure 4-2. Effects of Luminal and Intravenous Treatments on Mucosal Fractional Protein Synthetic Rates.

Weaned piglets were intravenously infused with glucose (20 mg . Kg^{-1} . h^{-1}, n = 6) or saline for 1 h. Within each infusion group, one of four intestinal segments constructed within a piglet was perfused with phosphate buffered saline (PBS, pH 7.3), or with 30 mM amino acid mixture plus 50 mM glucose, or 30 mM amino acid mixture, or 30 mM glutamine for 1 h. Protein synthesis was then measured as described in the text. Mucosal samples from perfused segments were processed as described under Materials and Methods. Analysis of variance revealed that, relative to intravenous saline treatment, glucose infusion significantly increased mucosal Ks ($P < 0.05$, n=6). Irrespective of intravenous infusion, luminal nutrients significantly suppressed mucosal protein synthesis relative to control PBS ($P < 0.05$, n = 12).

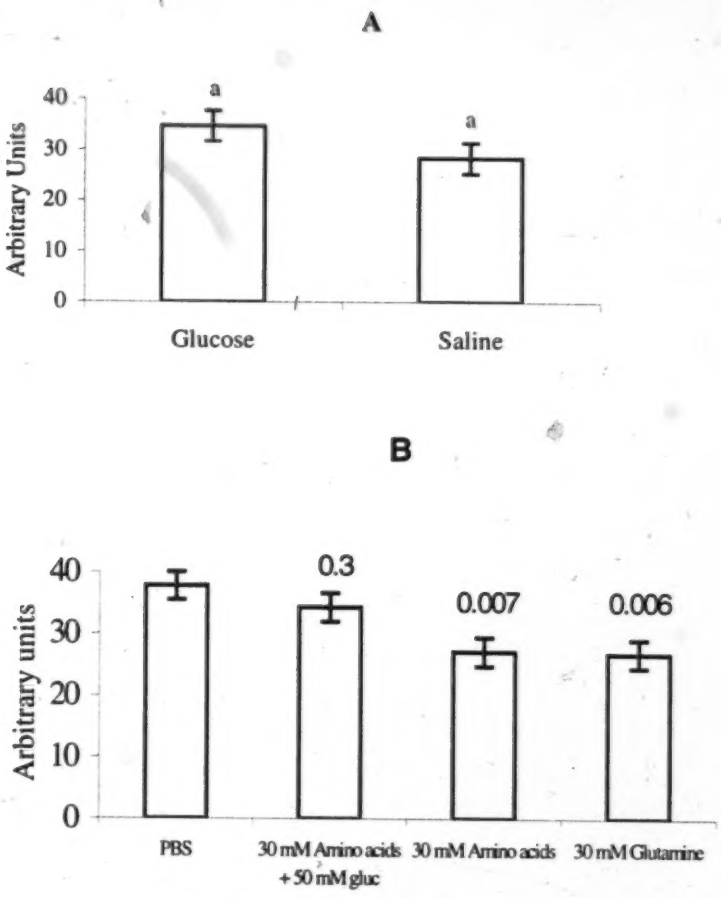

Figure 4-3. Effects of Luminal and Intravenous Treatments on Intestinal Mucosal Ubiquitin mRNA Levels.

Treatments were as described in legends to Fig. 4-2. Data are expressed as arbitrary units after scanning densitometry or phosphorylimage analysis. There were no significant effects of intravenous treatments (Fig. 4-3A, $P = 0.26$), therefore Fig. 4-3B represents pooled data of intravenous saline and glucose treatments. P values of differences from PBS are shown on the bars.

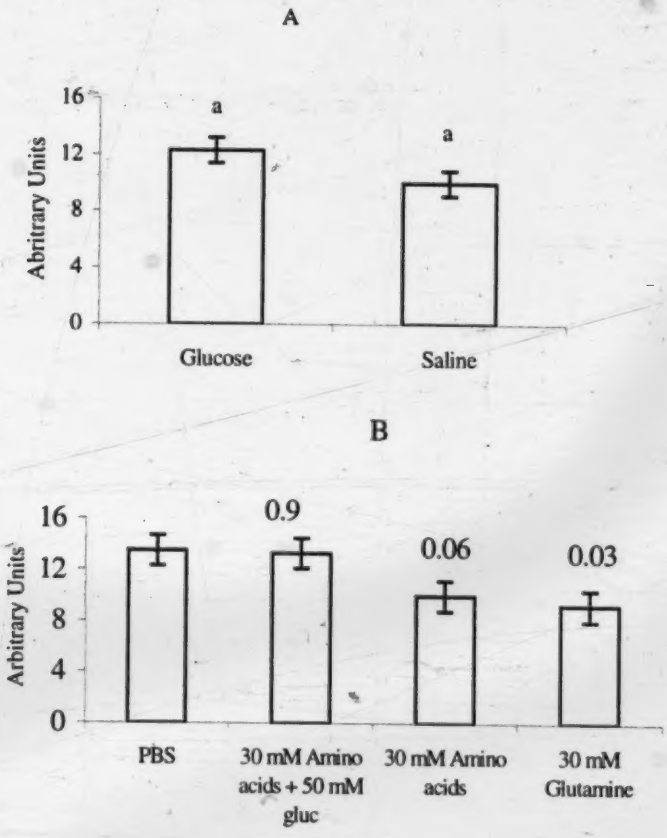

Figure 4-4. Effects of Luminal and Intravenous Treatments on Intestinal Mucosal mRNA Levels of 14 kDa E2 (Ubiquitin Conjugating Enzyme).

Treatments were as described in Materials and Methods. There were no significant effects of intravenous treatments (Fig. 4-4A, P = 0.11), therefore Fig. 4-4B represents pooled data of intravenous saline and glucose treatments. P values of differences from PBS treatment are shown on the bars.

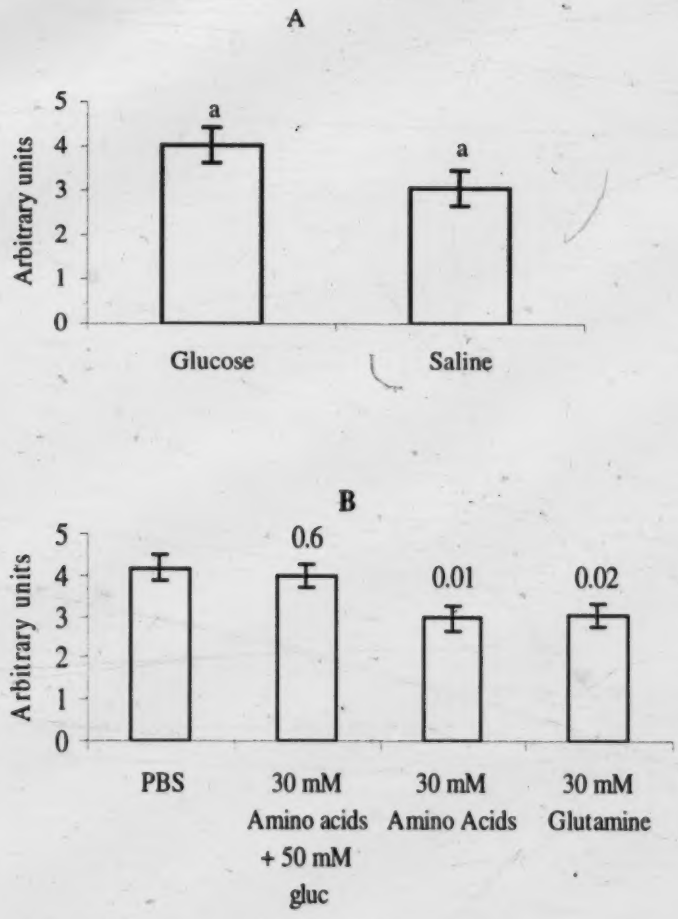

Figure 4-5. Effects of Luminal and Intravenous Treatments on mRNA Levels of C9 Subunit of the 20S Proteasome.

Treatments were as described in Materials and Methods. There were no effects of intravenous treatments (P = 0.11, Fig 4-5A) therefore Fig. 4-5B represents pooled data of intravenous saline and glucose treatments. P values of differences from PBS treatment are shown on the bars.

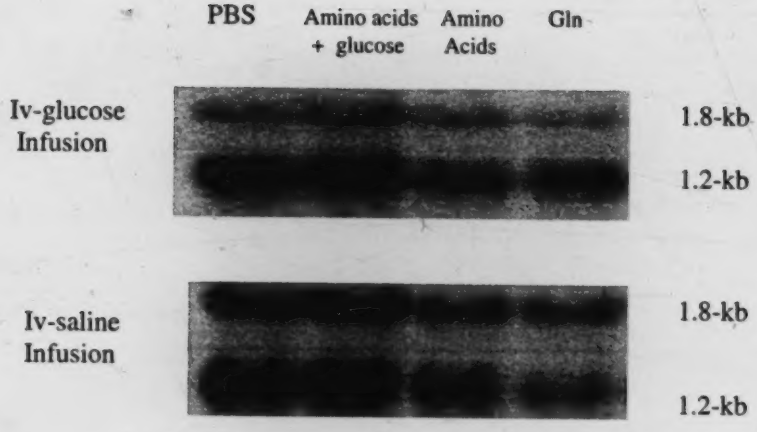

Figure 4-6. Representative Blots for Northern Hybridization Analysis of 14 kDa Ubiquitin Conjugating Enzyme.

Fifteen micrograms of total RNA from jejunal mucosal samples of piglets treated as described in Fig. 1 and Materials and Methods were separated electrophoretically in 1% agarose-formaldehyde gels. Northern hybridization analysis were carried out as described in Materials and Methods. Notice the intensity of 1.2-kb transcript relative to that of 1.8-kb. There were no significant effects of intravenous infusion but luminal perfusion of intestinal segments with 30 mM amino acids (Amino acids) or 30 mM glutamine (Gln) suppressed the expression of 14 kDa ubiquitin conjugating enzyme relative to PBS ($P < 0.05$, see Fig. 4-4).

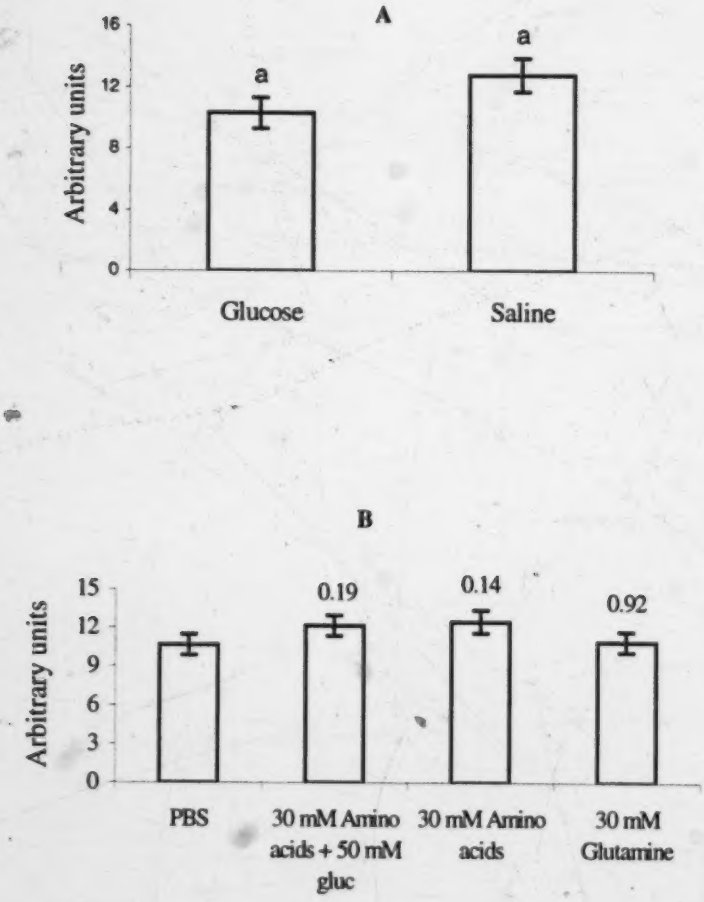

Figure 4-7. Effects of Luminal and Intravenous Treatments on mRNA Levels of m-calpain.

Treatments were as described in Materials and Methods. There were no effects of intravenous (P = 0.15) or luminal treatments (P values of comparisons to PBS shown on the bars).

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CHAPTER 5

General Discussion and Conclusion

The intestine was traditionally viewed as an organ that was mainly involved with digestion and absorption of nutrients, and that contributed little to body metabolism apart from its role in the supply of nutrients. Studies especially in this century have provided evidence that showed that this organ, in addition to its role as a digestive engine, plays significant roles in whole body glucose, amino acid and lipid metabolism under different physiological and pathological conditions (McBurney, 1993). It is now known to be an important endocrine organ (Drucker, 1997) and, especially under pathological conditions, to be able to mount immune responses (Perdue et al., 1994). Apart from the roles of the gut-associated lymphoid tissue in immune function, epithelial cells of the intestine, under pathological challenges, can produce cytokines as well as express major histocompatibility complex class II on their surfaces (Perdue et al., 1994).

Although the importance of the intestine in producing proteins involved in digestion and absorption of nutrients has long been recognized (see Alpers and Kinzie, 1973), Garlick and co-workers were the first to point to the significance of the intestine in whole body protein metabolism (Garlick et al., 1980; McNurlan and Garlick, 1980, 1981; McNurlan et al., 1979). They showed that although this tissue accounts for less than 7% of whole body protein content, it has the highest rate of protein turnover amongst the major tissues of the body, and contributes 19% of whole body daily protein synthesis in rats.

Subsequent studies have shown that the small intestine alone contributes 9-15% of whole body protein metabolism in rats and pigs (Alpers and Kinzie, 1973, McNurlan and Garlick, 1980, Simon et al., 1982).

Because of the importance of the intestine in digestion and absorption, and its contribution to whole body protein metabolism and growth, interest has been growing on the regulation of protein metabolism and growth of this organ under different physiological and pathological conditions. In the young animal for instance, there is a potential for growth that can only be realized with adequate nutrition (Davis et al., 1993, 1996). Also in human patients with conditions associated with anorexia or diseases of the intestine that make complete oral feeding impossible, total parenteral nutrition becomes the only source of alimentation. However total parenteral nutrition is associated with wasting of the small intestine and derangement in whole body metabolism (Burrin et al., 1994; Stein et al., 1994, Tappenden, 1997). Efforts are therefore being directed at understanding the regulation of the growth of the intestine and how this is affected by various hypercatabolic states such as trauma and diseases. In particular the importance of nutrition in the regulation of intestinal metabolism has received considerable attention.

The studies described in this thesis became necessary because our understanding of intestinal protein metabolism and the importance of nutrients in its regulation is very limited.

Methodology

Previous studies on the effects of nutrients on intestinal metabolism could not address the question in the manner it deserved. This is because many of the previous studies used

long term feeding trials making it impossible to separate the effects of luminal nutrients from those of systemic ones, and from the effects of hormones released in response to nutrient consumption (for example, see Ref. Dudley et al., 1998; McNurlan and Garlick, 1981; Wykes et al., 1996). In the first part of the studies therefore, a perfusion system that allows controlled and systematic studies of the acute effects of luminal nutrients on intestinal mucosal protein metabolism was described and validated. The methods present several advantages in studying intestinal metabolism. Because up to four intestinal segments can be perfused within the same animal, the system is cost effective. Comparison of the effects of different nutrients on mucosal protein metabolism is also rendered more sensitive as the effects of animal variations are reduced. In addition, the system can be used to answer questions relating to direct effects of nutrients on mucosal metabolism in a manner impossible with other methods. In comparison with feeding trials, which will unavoidably lead to elevated systemic levels of nutrients and hormones, the perfusion system described involved perfusion of only small segments (which constitutes 4% of small intestine surface area). Therefore within the short period of the perfusion, systemic concentrations of substrates in the perfusate were not changed by luminally perfused nutrients.

A further advantage of the system is the use of luminal flooding dose technique to measure protein synthesis. This technique is the method of choice in measuring protein synthesis because the short perfusion time ensures that none of the protein synthesized has been secreted and therefore synthesis of total protein is measured. The amount of tracer administered within a short time also ensures that specific radioactivities in all tissue pools are brought to the same level so that the use of intracellular free tracer pool

labeling approximates the true precursor (t_{RNA} pool) specific radioactivity (Garlick et al., 1980). However, this requirement for isotopes made the use of the technique for studying protein synthesis in larger animals impracticable (Scott et al., 1993). If one is interested in studying only intestinal protein metabolism it will be wasteful to label the whole animal. Hence the use of luminal flooding is a useful alternative which ensures that the requirement and cost of isotope used to label intestine is only about 10% of that which would otherwise be needed if the whole animal were to be labeled. In line with previous studies demonstrating that the intestine does not metabolize phenylalanine, one can circumvent the need to isolate phenylalanine from its metabolites. This was confirmed in the experiments described in Chapter 2. Thus, application of a luminal flooding dose technique further ensures that cost, time, and analytical errors associated with separation of phenylalanine from its metabolites are removed.

Multi-Segment Perfusion System and Luminal Flooding Dose Method

Issues discussed below arose in the course of experiments carried out using the multi-segment perfusion system. Addressing those issues will increase the potential of the system.

The perfusion of intestinal segments with amino acid mixture resulted in small changes in plasma concentrations of a few amino acids. This implies that using smaller animals such as rats for studies of the type described would be inappropriate because the length of intestine perfused would constitute a greater percentage of the intestine. Luminal perfusion with nutrients under such conditions would significantly raise plasma levels of the perfused nutrients as well as systemic concentrations of hormones and growth factors.

In studies with larger animals like piglets, reducing the concentrations of amino acids included in the perfusate or perfusing only one intestinal segment with amino acids could lessen the effects of luminal amino acids on systemic concentrations of perfused substrates. In addition, because the effects of nutrients on protein synthesis were seen by as early as 40 minutes (Chapter 3), the total perfusion time could be reduced to less than one hour. However, if changes in gene expression are to be measured, it will need to be ascertained that the degrees of changes desired could be obtained within the perfusion time chosen.

As discussed in Chapter 4, contrary to reports showing anti-proteolytic effects of glucose and insulin in muscle (Fryburg et al., 1995; Moldawer et al., 1980), glucose infusion (and associated elevated plasma insulin level) did not affect the expression of proteolytic genes within the gut mucosa. The main difference between this study and others was that insulin or glucose infusion was generally for 4 h or more. Therefore infusion times of longer than 1 h used in the studies described here may be needed for the effects of glucose and / or insulin on intestinal proteolysis to be seen. This can be done partly by starting the infusion immediately anesthesia is induced. The maximum time that piglets were kept under anesthesia in studies described in this thesis was 3 h (including surgery and perfusion time). Therefore, to conduct experiments for longer periods, it would be necessary to examine if the validation data shown in Chapter 2 still hold.

It was observed that when compared with animals that were intravenously flooded, mucosal free tracer (phenylalanine) specific radioactivity in luminally flooded animals was about 16% less two minutes after administration of tracer. Although the possible reasons for this have been discussed in Chapter 2, one point is worthy of emphasis. The

Km for phenylalanine inward transport at the brushborder membrane ranges from 2.7-4.7 mM compared to basolateral transporter with a Km of only 0.18 mM (Hu and Borchadt, 1992), indicating that the affinity of the basolateral transporter phenylalanine is several fold greater than that of the brushborder. Therefore, it might be possible to increase the degree of free phenylalanine labeling if higher concentrations of phenylalanine (chosen within the luminal physiological range) were used in the luminal perfusate. It should however be stated that although there were differences in free phenylalanine specific radioactivity at two minutes, these values were identical by 10 minutes. This was achieved because specific radioactivity in intravenously flooded segments had fallen while the values remained constant in luminally flooded segments. This advantage of luminal flooding (constancy of tracer specific radioactivity) is of greater significance and far outweighs the small differences caused by route of tracer administration at 2 min after isotope administration.

A further point relates to intracellular phenylalanine concentration during luminal flooding. Although free phenylalanine specific radioactivity changes very little with different luminal treatments, free phenylalanine concentrations were significantly reduced by amino acid treatments. This is to be expected because of competition between phenylalanine and other amino acids, especially the branched-chain and aromatic amino acids, for transport (Hu and Borchadt, 1992; McGivan and Pastor-Anglada, 1994). Although this difference in mucosal phenylalanine concentration can not be used to explain the effects of amino acids on protein synthesis (because mucosal free phenylalanine specific radioactivities were similar with the different treatments), the

effects of amino acids on mucosal free phenylalanine concentrations can be reduced by raising tracer (phenylalanine) concentration in the perfusate.

Nutrients and Jejunal Mucosal Protein Turnover

Using the validated multi-segment perfusion system, the acute effects of luminal and intravenous nutrients on mucosal protein metabolism were studied (Chapters 3 and 4). The main findings of these studies are

1. Luminal nutrients acutely regulate protein metabolism in intestinal mucosa by suppressing protein synthesis and decreasing mRNA levels of components of ATP-ubiquitin-proteasome proteolytic system. This suggests that luminal amino acids may regulate intestinal protein metabolism by mechanisms that reduce intestinal energy utilization (with respect to protein metabolism). This may ensure that a greater proportion of absorbed nutrients can be released to other organs in the body.
2. Intravenous glucose infusion increased mucosal protein synthesis by 16% but had no effect on the expression of proteolytic genes studied, nor was there any effect of intravenous treatments on the response of intestinal mucosal protein metabolism to luminal treatments.
3. The effects of amino acids in mucosal protein metabolism appeared to be dependent on luminal concentrations of the amino acids, rather than on specific amino acids such glutamine and its related amino acids (glutamate, arginine, and proline). Glutamine alone at the concentration found in the amino acid mixture had no effects on protein synthesis. Also, when glutamine,

arginine, glutamate and proline were deleted from the mixture, the suppressive effects of amino acids on protein synthesis were still seen. This was not surprising as studies showing beneficial effects of glutamine on intestinal weight, DNA content and protein synthesis metabolism did so only when the effects of glutamine were studied in diseased, traumatized or catabolic animals (Burrin et al., 1991; Garcia-Arumi et al., 1995).

Although it has been little studied, protein degradation appears to be a very important component of intestinal protein metabolism. The study of Samuels et al. (1996) demonstrated that after one day of fasting in adult rats, although protein synthesis decreased insignificantly by 10%, expression of genes involved in proteolysis decreased by between 40 and 200%. These changes in proteolytic genes thus paralleled the protein loss (20%) observed in intestine more closely than protein synthesis. Results of experiments described in Chapter 4, where changes seen in protein synthesis in response to luminal amino acids were about half the magnitude of the changes in expression of proteolytic genes, are also in support of the importance of proteolysis in intestinal protein metabolism. If the same kind of regulation applies in situations associated with intestinal wasting such as diseases and feeding with total parenteral nutrition, then in addition to nutrients, adjuvants and drugs that are known to suppress proteolysis need to be studied to see if they offer any protective effects against protein catabolism in the intestine.

A final note relates to tissue samples harvested after different perfusion experiments. As detailed in Chapters 2 to 4, intestinal mucosa was scraped from perfused segments and analyzed for rate of protein synthesis. The intestinal mucosa contains not only absorptive

epithelial cells but other cell types as well (for example, immune and endocrine cell types). We did not determine the contribution of the different cell types to the observed effects. Park et al. (1994) measured protein synthesis in lymphocytes (isolated from the blood) and showed that in healthy subjects, the fractional rate of protein synthesis was about 9% / day. This however increased to 28% upon infusion of interleukin 2. Since healthy piglets were used in our experiments and because endocrine and lymphoid cells constitute, respectively, 1 and 15% of the mucosal cells (Elson and Beagley, 1994, Hermiston et al., 1994), the observed responses of intestinal mucosa to luminal nutrients in this work can be attributed largely to mucosal epithelial (absorptive) cells. However, even the villous epithelial cells are a mixture ranging from fully differentiated cells at the tip of the villi to immature cells in the crypt. Because protein metabolism in the different cell types as well as amongst cells with different degree of differentiation may be regulated differently (Higashiguchi et al., 1994), the observed effects of luminal and intravenous nutrients on mucosal protein metabolism represent an aggregate response.

The effects of the different treatments on specific intestinal proteins were also not studied. In a recent study with neonatal piglets, absolute synthetic rate of total mucosal proteins was diminished by intravenous feeding. However the absolute synthetic rate of lactase phlorizin hydrolase, an enzyme involved in the hydrolysis of milk lactose, was not affected by the route of feeding (Dudley et al., 1998). Therefore metabolism of specific proteins may be differently regulated from that of total mucosal proteins. Since specific nutrients are known to regulate the activities (and probably synthesis) of proteins involved in their digestion and absorption (Stevens, 1992; Cheeseman, 1991), one may speculate that digestive enzymes and nutrient carrier proteins are regulated in the

absorptive state such that the digestion and absorption of nutrients are maximized. Further studies will be needed to examine how the metabolism of distinct intestinal proteins is regulated by nutritional influences in the absorptive state.

Future Direction

1. Measurement of Protein Degradation in Intestinal Segments

A further extension of the luminal perfusion system is its application in estimating intestinal proteolysis. This can be approached by using the 'pulse-chase' technique (Crissman et al., 1994). The test solutions are perfused as described in Chapters 3 and 4. Luminal flooding dose is then used to measure protein synthesis. To measure protein degradation, in another sets of piglets, the intestinal segments previously perfused with labeled tracer having high specific radioactivity (pulse) will be perfused with cold solution of tracer (chase) and the disappearance of radioactivity in labeled- protein followed. The chase solution in each segment will also contain the solutions whose effects on proteolysis are being examined. To account for protein secretion into the intestinal lumen, the perfusate is collected and analyzed for protein-associated radioactivity. If fasted animals are used, protein secretion into the plasma during the period of the chase should be minimal. Perfusion with cold (non-radioactive) tracer should minimize label recycling. However, to further account for the extent of label re-utilization, protein-associated radioactivity in adjacent unperfused intestinal segments can be measured. Since the only source of radioactivity in unperfused segments is the plasma, such measurements will help in estimating the amount of label released from protein degradation that are re-incorporated into tissue proteins.

By including chemicals / drugs that are known to be specific inhibitors of different proteolytic pathways in the perfusate during the 'chase', the contributions of different proteolytic systems to intestinal proteolysis can be estimated. The appropriate inhibitors (see Chapter 1 of this thesis; Gronostajski et al., 1984, 1985; Baracos et al., 1995) would be:

ATP-dependent Proteolytic Pathway: NaF or 2-deoxyglucose (chemicals that reduce ATP production) and Rotenone, Dinitrophenol, or NaN_3 (inhibitors of oxidative phosphorylation).

Studies with these inhibitors will give an estimate of the contribution of total ATP-dependent, but not necessarily ATP-ubiquitin-proteasome dependent, proteolytic pathway to intestinal proteolysis. Some proteins, notably ornithine decarboxylase, an important intestinal mucosal protein, are degraded by the 26S proteasome but without ubiquitin conjugation (Murakimi et al., 1992).

Lysosomal Proteolytic System: NH_4Cl, Chloroquine, Methylamine, Leupeptin, EP475, Pepstatin;

Ca^{2+}-dependent Proteolytic System: Dantrolene, Leupeptin.

A series of preliminary experiments will be needed to examine the optimum conditions for such experiments and the possible effects the different inhibitors might have on intestinal metabolism (apart from their effects on proteolysis). For example, the drugs to

be used should not affect the inward transport of the 'chase' amino acid, and of other amino acids and nutrients whose effects are being tested. Previous studies with skeletal muscle have shown that ATP depletion can cause Ca^{2+} accumulation in the cytosol and activation of the Ca^{2+}-dependent proteases (Han et al., 1988). Therefore perfusate containing drugs / chemicals that cause ATP depletion must also contain inhibitors of the calpains.

2. Relevant Physiological Questions

Studies described in this work lead to arrays of physiologically relevant questions. By what mechanisms do luminal nutrients suppress expression of proteolytic genes? For instance, are the observations made limited to luminal nutrients alone, or will similar observations be made if amino acids were delivered intravenously? The effects of intravenous glucose infusion, was it due to insulin, glucose, or both? How will hormones and growth factors alter the response of intestine to luminal nutrients? Luminal nutrients suppressed both protein synthesis and degradation. By what mechanisms were those changes mediated? What kinds of proteins are involved? What will be the response of intestine to these nutrients under catabolic and diseased states such as lactation, cancer, intravenous feeding (TPN), and chemotherapy? Glutamine and ornithine α-ketoglutarate have been shown to promote intestinal and whole body protein anabolism under catabolic states such as tumor, surgery, and TPN-feeding. Do these nutrients have direct effects on intestinal protein metabolism, and if yes, by what mechanisms? In livestock production, weaning is associated with a number of intestinal–related abnormalities such as diarrhea and derangement in protein metabolism, leading to reduced performance and sometimes

death of piglets (van Beers-Schreurs et al., 1992; Wu et al., 1996). Do luminal nutrients have any role to play in reducing these effects? Answers to these questions will help not only in improving human welfare but also in increasing livestock production as well.

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Appendix 1

Luminal Flooding Dose Technique

SURGERY

Requirements:

Personnel: Need at least three people for a hitch-free operation

Drugs and Anesthetics:

Pre-Med Cocktail: Torbugesic (Ayerst Laboratories, Montreal Canada), Rompun (Miles Canada Inc., Etobicoke, Ontario Canada), Glycopyrrolate (Sabex Inc. Boucherville, Quebec Canada), Ketamine (MTC Pharmaceuticals, Cambridge, Ontario Canada).

Others: Halothane, Euthanyl (for killing the animals after experiments: MTC Pharmaceuticals, Cambridge, Ontario Canada

Equipment:

Anesthetic Machine, Water Bath, Rectal Temperature Probes (e.g., Temp probe with Model Bat 8 From Bailey Instruments, Saddle Brook, NJ), Gas Masks, Surgical Heat Mat, Over-Head Infra Red Lamp, Pump for luminal perfusion (Cole-Parmer Instrument Chicago, IL, Model # 7619-40), intravenous infusion pump (Peristaltic pump, Pharmacia Fine Chemicals Cat. # 19-0626-01).

Materials:

Surgical blades, scalpels, and scissors; gloves, umbilical tapes (Braided umbilical tape, Baxter Healthcare Deerfield, IL, Cat. # 42520-010), Gauze (10x10cm, Dumex Scarborough Ontario Cat. # 90412),

Polyethylene tubes as cannulas (inlet cannula: internal diameter; 1/8 inch, outer diameter, 3/16, wall, 1/32"; outlet cannula: 3/16, 5/16, 1/16" for internal, outer diameters and wall, respectively: Fisher (Tygon); R-3663, 14-169-1),

Funnel (internal diameter 6.5 cm), Connectors and 3-way stop cock (Medex, Hilliard, Ohio, Cat. # MX493 and # MX411, respectively),

60-mL syringes, ice bucket, plastic containers filled with saline and placed on ice, liquid nitrogen, labeled sampling vials, plastic surface and microscopic slide for scraping the mucosa (these were immersed in ice)

Solutions:

Sterile Pyrogen-free Saline: (Baxter Cat. # W7D17B3) For intravenous infusion.

Phosphate-buffered Saline (PBS); 1L

(339 mosmol/L)

NaCl	8.45g
Na_2HPO_4 (M. Wt 142)	2.26g
$NaH_2PO_4.H_2O$ (M. Wt. 138)	0.161

Saline: 0.9% NaCl for rinsing intestinal segments after harvesting them from the animal at the end of the experiment.

Isotope Solution

Make 500 mL of 2 mM L-phenylalanine solution in PBS:

You can make 150 mM stock. To do this, dissolve 2.478 g in 100 mL PBS. (You will have to heat this solution to dissolve). Cool, filter (e.g., 0.2 μ Millipore filter), store frozen if for longer use, or at 4°C. Then dilute as needed.

To 500 mL 2mM phe, add 330 μCi of L-[2,6-^{3}H]phenylalanine (Amersham, Code TRK.552). This would give a specific radioactivity of ~730 dpm / nmol phe. If the isotope has been in store for sometime (say 6 months to 1 yr), activity remaining can be got from the formula

$$A_t = A_o (0.5)^{t/T_{0.5}}$$

where A_t = activity remaining at time t; A_o = initial activity at time t = 0,

$T_{0.5}$ = half-life for the isotope ($T_{0.5}$ for ^{3}H =12.3 yr); t = elapsed time.

Surgery

Overnight-fasted piglets were used. Next morning, piglets were brought into the surgery room and injected intramuscularly with a cocktail of pre-med: Torbugesic (0.2 mg/kg), Ketamine (11 mg/kg), Rompun (2.2 mg/kg) and Glycopyrrolate (0.01 mg/kg).

While waiting for the pre-med to take effects, umbilical tapes of length 10, 15, and 50 cm were measured out. Another set of eight (20 cm) were measured out. Fifteen cm is the length that is measured of from the peritoneal inflection to the insertion of inlet cannula of the first segment; 10 cm is the length of each intestinal segment; 50 cm is the length between adjacent segments; the other 8 pieces (20 cm) will be used to tie the intestine around the cannula (one for inlet, one for outlet; therefore for 4 intestinal segments, 8 will be needed). After measuring out the segments and before the cannulas were put in place, it proved difficult at times to know which were the tapes for inlet or outlet cannulas. Therefore, the tapes for tying each segment were marked with numbers corresponding to the segment (i, ii, iii, iv), and were marked red or blue to indicate which were inlet and which were outlet tapes.

After the drugs had taken effect (about 15 minutes after injection), the animal was place on the operating table and its legs tied to the four corners of the table, care being taken not to cut off blood supply to the legs. With the aid of a face mask, the animal was then hooked up to the anesthetic machine which delivered 3L / min O_2 with 2% Halothane (see picture, Appendix 1A). The animal was heated throughout the experiment with a surgical, water-heated blanket wrapped round the animal. An over-head infra red bulb was used to provide additional heating as required.

The skin covering the abdomen was shaved and cleaned with alcohol. With the aid of a scalpel blade, a midline incision was made. To locate the jejunum, the intestine was held gently and traced posteriorly until the peritoneal inflection was located (this is the point at which the intestine coils around its own axis). The intestinal segment from the end of this coil to the ileo-cecal junction represents the jejunum (proximal half) and the ileum (distal half). To ensure that one was working with the jejunum, the inlet cannula of the first segment was inserted 15 cm from the beginning of the jejunum. Intestinal length between

inlet and outlet cannulas (corresponding to segment length) was 10 cm. Four 10-cm intestinal segments were cannulated at both ends (inlet at the pyloric end, outlet at the ileal end) with polythethylene tubing (tube diameters: Internal Diameter (ID) = 1/8 and 3/32", OD = 3/16 and 5/16", respectively for inlet and outlet cannulas, see Plate 1-1 in Chapter 2). Successive segments were separated by 50 cm of intestine Before the cannulas were put in place, the four segments were first measured out using umbilical tapes pre-measured to the desired length. Care was taken to ensure that each segment had its own well-defined vascular system. This was readily verified as the intestinal segment rapidly became pale if its blood vessels were tied off.

To insert the cannula, with the tip of a new scalpel blade, a small incision was made on the antimesenteric side of the segment. A small pair of scissors, with the tips closed, was then gently inserted into the opening made with the scalpel. The tips of the scissors were then gently opened so that enough space was made for the insertion of cannula with as little bleeding as possible (blunt dissection). The cannulas were then inserted and tied in place. At all times, care was taken not to mangle the intestine. Note that the internal diameter of the outlet cannula was greater than that of the inlet ones (see above). This was chosen so as to ensure easy draining of the intestinal segments during the perfusion. To ensure that the cannulas stayed in place, their ends were fitted with a small funnel. The top ends of 6.5-cm diameter funnels were cut so that the internal diameter was reduced to 1.5 cm (see Fig A-1.1). These were then inserted into the ends of the inlet and outlet tubings. It was the funnel ends of the tubing that were inserted into the intestinal segments. In this way, it was easy to tie the intestine over the cannula. Also, we had minimum incidence of cannulas slipping out of the segments.

After insertion of cannulas, intestinal segments were rinsed of digesta remnants with warm PBS (using a 60-mL syringe). The intestinal loops were kept moist by spraying with warm PBS and were covered with PBS-soaked gauze and transparent polythethylene nylon to reduce evaporation. Each of the four segments had its own separate perfusion line connected to a multi-channel pump.

To measure protein synthesis, 2 mM ^{3}H-phe solution was used. The volume of a 10-cm segment was about 5 mL. But the length of the perfusion line was chosen so that the

entire volume of perfusate in the perfusion line was 50 mL. This was to ensure that during the 15 min of isotope perfusion, [phe] would not fall appreciably. The total amount of phe in line was 100 μmoles (ml X [phe] = 50ml X 2 mM = 100μmoles phe).

Luminal Flooding (Fig. A-1.2 and A-1.3)

During surgery, the polyethylene tube length labeled C (about 5.6 m) in Fig. A-1.2 below was filled with warm (37°C) perfusate. The two ends of section C were placed in the solution positioned at B (labeled perfusate) and the pump was kept running. This ensured that section C was filled with warm (37°C) isotope solution (Step 1, Fig A-1.3). The outlet end of C was put in the perfusate container to prevent wasting of the perfusate (Step 1 of Fig. A-1.3). To start perfusate perfusion, one person was responsible for filling section A with warm (37°C) isotope (Fig. A-1.2 and Step 2 in Fig. A-1.3). This section consisted of the intestinal segment with its inlet and outlet cannulas. Another person was the time keeper while a third person was responsible for connecting loaded intestinal segments (A) to their respective perfusion lines (i.e., connecting the inlet end of A to D and the outlet end of A to B (see Fig A-1.2 and Step 3 of Fig. A-1.3). To synchronize perfusion as much as possible, four 60-mL syringes were loaded with warm isotope solution and were each used to fill one intestinal segment. The person responsible for loading filled the first intestinal segment with the isotope from the 60-mL syringes. At the same time, he told the recorder to note the time. When the segment was filled, the third person rapidly stopped the pump, connected the segment to its inlet and outlet lines and restarted the pump (Step 3 of Fig A-1.3). The cycle was repeated for the second intestinal segment, with the time at which perfusion of each line started being noted. Thus, the four segments were perfused.

After the first intestinal segment had been perfused for 15 minutes, the segment was carefully dissected and immediately immersed in ice-cold saline. The time of immersion in saline was taken to be the end time of perfusion. Other segments were sequentially taken out and immersed in cold saline after perfusion of each for 15 minutes. The segments were flushed with cold saline and, while still being kept in saline, cut open along the mesentery. The intestine was then spread on a cold surface and mucosa rapidly

scraped with the aid of a microscope slide and put in labeled container which in turn was rapidly immersed in liquid N_2. Samples were stored at –50°C or –80°C.

After the removal of perfused intestinal segments at the end of the experiments, animals were killed by cardiac injection of Euthanyl (pig: 1 ml/kg body weight).

Sample Analysis

Approximately 300 mg of mucosa from each of the segments was powered in liquid N_2 and then homogenized in 3mL of ice-cold 2% perchloric acid. Samples were then centrifuged at 2, 800 x g for 15 minutes. Supernatants were collected and neutralized with half volume (~1.5 mL) saturated tri-potassium citrate and stored frozen until ready for analysis. Pellets were washed four times with 8ml ice-cold perchloric acid, each time with centrifugation at 3,000 x g. Washed pellets were hydrolyzed in 5 mL 6 N HCl at 110°C for 24 h. Excess HCl was dried off under vacuum. The dried pellet was then resuspended in 1.5 ml of 0.5M trisodium citrate (14.71g trisodium citrate dissolved in 100 mL ddH_2O, pH to 6.3 with 10 M HCl). On both supernatant and pellet fractions, specific radioactivities of phenylalanine were determined and were referred to as free- and protein-bound phe specific radioactivities. These determinations were carried out as described in Chapter 2 and in references therein.

Effects of Nutrient Solutions on Protein Synthesis

To examine the effects of different luminal nutrient solutions on mucosal Ks, cannulation and perfusion were carried out as described above. However, the four intestinal segments were first perfused with the desired solutions for 40 to 90 minutes. The segments were then emptied and filled with isotope. To do this, four additional perfusion lines were included. These contained only the section labeled C in Fig. A-1.2. Thus, there were two sections C but only one section A for each intestinal segment (i.e., tow lines depicted in Step 1 Fig. A-1.3 for each segment). All the eight perfusion lines were pumped by a single pump (with 8 channels). Four of the lines were used to perfuse the test solutions(that is, the solutions, such as amino acid or glucose, whose effects on mucosal protein synthesis are being examined), and four for isotope. When it was about 20

minutes to the start of isotope perfusion, the section labeled C (Fig. A-1.2 and Step 1 of Fig A-1.3) for the second set of four lines was connected to the pump and filled with isotope as described above so that by the time isotope perfusion would start, these lines would already be filled with isotope. To start isotope perfusion, the C section of the four lines used to perfuse cold treatment solutions were disconnected from section A. Remaining cold solutions in sections A were gently but thoroughly pushed out with air using 60-mL syringes. Sections A were then filled with isotope, connected to section C and perfused as described above.

Fig. A-1.1: Perfusion Cannulas

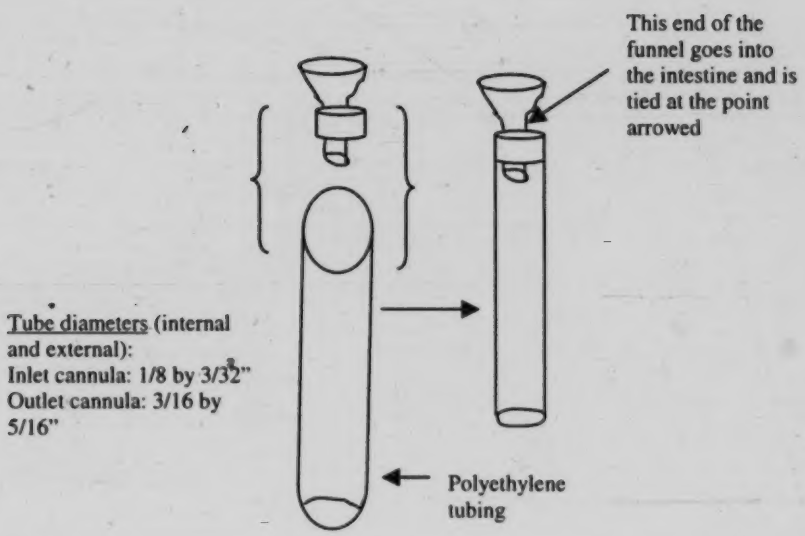
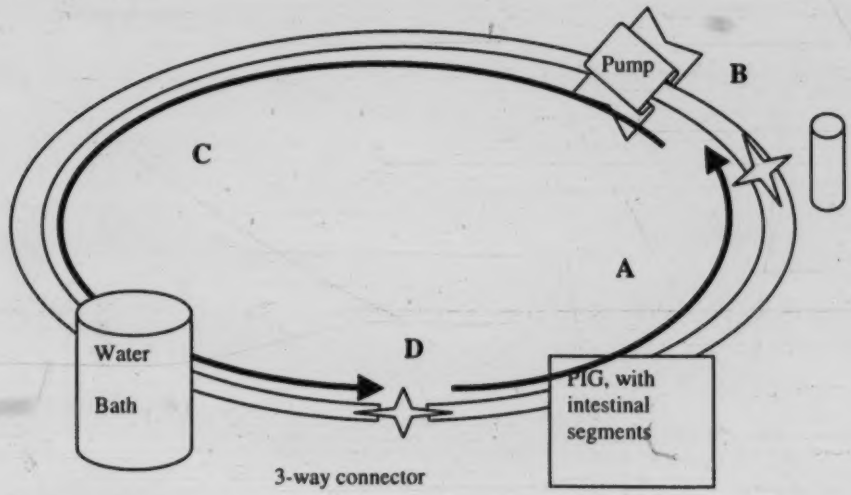

This end of the funnel goes into the intestine and is tied at the point arrowed
Tube diameters (internal and external):
Inlet cannula: 1/8 by 3/32"
Outlet cannula: 3/16 by 5/16"
Polyethylene tubing

Fig. A-1.2: Schematic diagram of the entire set up. See plate 2 for more details. Note that section C and the inlet end of A have the same diameter. Section of A from intestine to the connector at B is the outlet cannula.



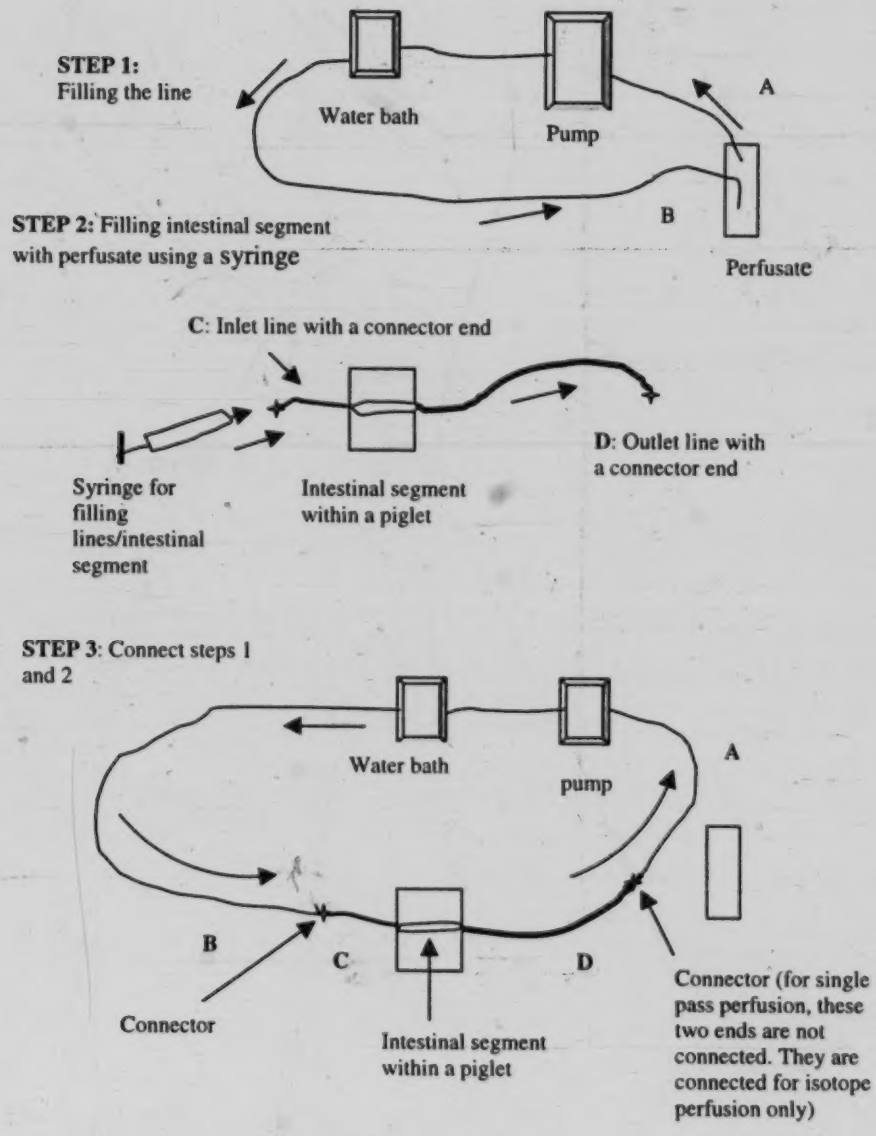


Figure A-1.3. Steps Involved in Loading the Perfusion Line and Intestinal Segment with Perfusate

Appendix 1A. Set up of the experimental area
1. Water bath; 2. Anesthetic gas line; 3. Face mask for delivering oxygen and anesthesia mixture; 4 Inlet lines going into the intestinal segments;
5. Pump for perfusing solutions through the lines

Apeendix 1A. Set up of the Experimental Area.

1. Water bath; 2. Gas mask for delivering oxygen and anesthesia mixture to the animal; 3. Anesthetic gas line; 4. The inlet lines going into the intestinal segments; 5. Pump for perfusing the solutions through the lines.

Appendix 2

Northern Hybridization

TOTAL RNA ISOLATION, ELECTROPHORESIS AND RNA TRANSFER

RNA ISOLATION

RNA isolation is done using TRIZOL™ Reagent (Cat No 15596, Life Technologies) according to manufacturer's instructions. The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate.

Care must be taken to guide against RNAases. Because their activity is difficult to inhibit, take preventive measures by

Wearing disposable gloves at ALL times when working with RNA

Using STERILE disposables. Plastic wares must be rinsed with 0.1N NaOH for 20 minutes, rinsed with sterile deionized distilled water (ddH_20) and autoclaved before use.

Baking ALL glass wares (overnight at 180°C).

Tissue samples for RNA analysis must be frozen immediately in liquid N_2 after removal from the animal, and must be stored at –70°C.

In addition to Trizol reagent, the following are needed:

Chloroform (without any additives (BDH, Cat No ACS210)

Isopropyl alcohol (Fisher, Cat # 405-7)

75% ethanol (in sterile water), 14 mL falcon tubes (Becton Dickinson # 4-2059-1, Lincoln Park NJ)

Disposable 5- and 10-mL sterile serological disposable pipettes (Fisher)

1 mL pipette and sterile tips, 1.5 mL microcentrifuge tube

1) Mix frozen mucosal samples with liquid N_2 and grind with mortal and pestle on dry ice.

2) Weigh 200-300 mg mucosal samples into 14-mL falcon tubes.

3) Homogenize tissue sample in Trizol, using 1 mL per 100 mg of tissue. Sample should not be greater than 10% of the volume of Trizol. Homogenize until no lumps are visible (Polytron homogenizer, setting 8 for ~ 3 min)

4) Incubate homogenized samples for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes. Add 0.2 mL chloroform per mL of Trizol used in step3 above.

5) Cap securely and shake vigorously by hand for 15 seconds. Incubate at room temperature for 2-3 min. Centrifuge at 12,000g for 15 min at 4°C. The mixture separates into an upper aqueous phase containing the RNA (~60% of volume of Trizol) and a lower red organic phase with an interphase in between.

6) Carefully transfer the aqueous phase into a fresh tube. Add 0.5 mL isopropyl alcohol per mL of Trizol. Incubate at room temperature for 10 min and centrifuge as in step 5 above for 10 min. RNA is seen as a gelatinous precipitate on the side and bottom of the tube.

7) Remove the supernatant, wash the pellet once with 75% ethanol, using at least1 mL 75% ethanol to 1 mL of Trizol. Mix the sample by vortexing and centrifuge at 7,500g for 5 min at 4°C.

8) Remove as much of the alcohol as possible. The manufacturer recommends that the RNA should not be allowed to dissolve completely as this would affect its solubility, which in turn would make RNA samples to have $A_{260/280}$ ratio <1.6. However, it was observed that remnant alcohol makes subsequent pipetting difficult. As much of the alcohol as possible was removed, and to dissolve the samples, samples were suspended in ddH_2O and vortexed.

9) Dissolve RNA in ddH_20. It is important not to use too great a volume of water so that between 5-10 µl of final solution can be used for electrophoresis (see below). As a test, one or two representative samples can be solubilized first and absorbance taken. Other samples can then be solubilized based on the estimate of sample concentration. Generally, when 250-300mg wet mucosa were used, the final pellets were dissolved in 200-250µl ddH_20.

10) RNA CONCENTRATION: Three to five microliters of re-suspended RNA samples were combined with 1 mL water in triplicates and the absorbance read at 260nM. At the same time, RNA purity was checked by examining the ratio $A_{260/280}$. The ideal ration is 2.0; values less than 1.6 are undesirable. Lower ratio may be due to **a)** A small volume of reagent being used, **b)** Contamination of aqueous phase with the organic phase, **c)** Incomplete dissolution of the final pellets.

11) Because it is not desired that the pellet be subjected to repeated cycles of freezing and thawing, RNA concentration should ideally be done just before electrophoresis (24-48 h before electrophoresis). We typically got a concentration of ~3 µg RNA/ mg wet mucosa.

12) After homogenization and before adding chloroform, samples can be stored at –70°C for at least a month. The RNA pellet in 75% ethanol(step 7) can be stored at 4°C for a week or a year at –20°C.

13) Troubleshooting:

Low yield: incomplete homogenization or lysis of samples, Incomplete dissolution of final pellet

RNA Degradation:

Tissue not frozen immediately or not stored at –70°C

RNA not stored at –70°C, RNAase contamination

Formaldehyde used for agarose-gel electrophoresis had a pH < 3.5

RNA GEL ELECTROPHORESIS

Chemicals: **MOPS** (3-(N-Morpholino)Propane Sulfonic Acid, $C_{17}H_{15}NO_4S$ (F Wt 209.26, Fisher, Enzyme Grade, Cat #BP308-500); 37% Formaldehyde; Sodium acetate (Sigma, anhydrous); NaOH; EDTA, Sodium Citrate; Ethidium bromide (10mg / mL stock, Life Tech., Cat # 15585-011); Glycerol; Bromophenol blue (BFB, $C_{19}H_9Br_4O_5Sna$, F Wt. 691.9, Sigma Cat #B5525); deionized Formamide; Agarose (Ultra Pure, Life Tech. Cat #15510-019

Materials: 0.22µm size filter (e.g., Millipore Sterivex-GP , Cat # SVGB 1010); Electrophoresis Unit, Electrophoresis Power Supply Unit (Tyler Research Instruments, Canada, Model EPS 200/250) Scientific

SOLUTIONS

37% Formaldehyde

10 X MOPS

To make 1L

0.20 M MOPS	41.86g
0.05M Sodium acetate	6.80g
10 mM EDTA	3.72g (20 mL of 0.5M EDTA)

Bring the pH from 5.5 to 7.0 with 10N NaOH.

Do not autoclave. Filter with 0.22µm size Millipore Sterivex-GP filter unit.

Store at room temperature in a light-proof container.

20 X SSC

175.3 g NaCl

88.2 g Na Citrate (trisodium) (MW 294.1)

qsp ~800 mL autoclaved ddH_2O

Adjust to pH 7.0 with 6 N HCl

qsp 1 L

autoclave 20 min and cool

adjust volume with autoclaved ddH_2O.

Loading Dye

Ethidium bromide is carcinogenic in contact with skin. Never handle with bare hands. Use only appropriately labeled disposal bags

160 µl 10 X MOPS

175 ml sterile water

25ml ethidium bromide (10mg / mL stock)

80 µl glycerol

80 ml saturated bromophenol blue (BFB) crystal in sterile water (add a small amount of BFB crystals to a microcentrifuge tube and vortex, centrifuge briefly). Before use, mix very well, then centrifuge briefly.

Solution is stable at room temperature if kept in a light-proof container. Always mix well before use.

Sample buffer

This buffer should be made fresh just before use

48 ml deionized formamide; **17.3** ml 37% formaldehyde (in a fume hood), **34.7** ml loading dye (above).

Procedure

Set up the Electrophoresis Unit: Soak the electrophoresis unit comb and the bridges in 0.1 NaOH for 10 min. If there is the possibility that the electrophoresis unit itself might have been contaminated with RNAase, it too should be soaked in 0.1N NaOH. Drain and dry. Gently put the comb in place, in a straight line, parallel to the width of the electrophoresis unit. Take care to ensure that the edge of the comb is neither too far from, nor touch the surface of the plastic stand in the unit. Seal the ends with the bridges. In setting up, use combs of the right size depending on the volume of samples to be loaded in each lane: the larger the volume, the larger should be the size of the comb edges. Alternatively, the volume of the wells can be controlled by varying the amount of agarose made.

Melt 1.4 g agarose in 120 mL of sterile water in a flask in a microwave oven. Take care that the agarose does not boil over.

Cool down to 55°C

Add 14 mL of 10 x MOPS

Add 7.6 mL of formaldehyde in fume food

Mix gently to avoid any air bubbles.

Gently pour the agarose into the gel electrophoresis unit, with sealed ends and a comb that does not touch the surface of the plastic stand. Avoid bubbles.

Allow the gel to solidify for 30-60 min. After the gel solidifies but BEFORE loading samples, cover the solid gel with 1 X MOPS (dilute 10 X MOPS: 900 mL ddH_20, 100 mL 10 X MOPS).

While waiting for the gel to solidify, add 10 µl of sample loading buffer and vortex to dissolve the RNA. Boil the mixture for 2 min in a heating block filled with water. When the samples have started to boil, reducing the flame intensity (by reducing the gas flow rate) can help in preventing the sample vials from opening.

Chill the tubes on ice. Centrifuge the tubes briefly to collect condensation and load immediately into the gel, noting the positions of each sample.

Turn on power to the unit. Run the electrophoresis at 100 Volts or less until the bromophenol blue reaches the end of the gel (about 5 h). During the electrophoresis, different sizes of RNA will migrate from the –ve end of the unit to the +ve end. So after running the gel for about 20 min, check to make sure the bands are heading the right way.

After running for desired time, remove the gel from the unit and place in 10X SSC. Mark it for the position of samples.

(UV LIGHT IS HARZADOUS. WEAR PROTECTIVE COVERINGS FOR EYES AND AREAS OF THE BODY THAT MAY BE EXPOSED).

Examine the gel with UV illumination using UV transilluminator or the Gel doc system. Take a picture alongside a ruler, its O point placed at the wells (origin).

Do not allow the surface of the gel to dry prior to transfer (see below).

Transfer of RNA to Nylon Membranes (Genescreen, NEN, Boston, MA)

Materials: GeneScreen Nylon membrane (NEN Life Sciences, Boston MA Cat. #NEF972); Filter Paper, Paper Towel.

Trim the gel at the edges to remove unused parts so as to reduce the amount of membrane needed.

Soak the gel in 5 volumes of sterile water for 5 min to remove the formaldehyde from the gel. Repeat 4 times.

Cut the GeneScreen membranes to the exact size of the membrane. Cut another 3 pieces of filter paper to the size of the membrane. Then cut a pile of paper towels to the size of the membrane.

Place membrane in distilled water for a few seconds until fully hydrated.

Soak membrane in 10XSSC buffer for 15 minutes.

Set up capillary blot using 10XSCC as the transfer solution. Place gel face down on a stand placed inside the capillary container that contains transfer buffer. Place and align membrane on the gel. Place the filter paper pieces and then enough pile of the cut paper towel. Place a small weight on top of the paper towel to increase contact through the pile. BE SURE TO REMOVE AIR BUBBLES between the filter paper set, the gel and the membrane. Also, use enough paper towel so that the top of the pile is still dry by the time the transfer is complete.

Transfer for **16-24 h**, changing paper towel layers and adding more buffer as necessary.

After transfer, carefully remove the filter paper **without** disturbing the membrane.

Carefully lift the membrane away from the gel with plastic forceps

Rinse the membrane briefly in 2XSSC to remove residual agarose. Place the membrane on a piece of paper to dry.

Fix RNA to membrane using UV crosslinking.

UV cross-linking:

Cross link the membrane while wet (damp)

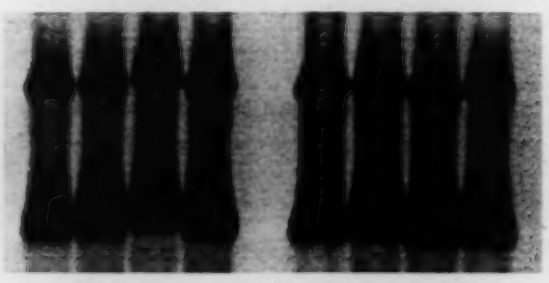
After transfer is complete, rinse membrane in 2XSCC to remove residual agarose. Any residual agarose on the membrane will lead to high autoradiographic background.

Place the wet membrane with RNA side facing up on a piece of wet filter paper. This will prevent the membrane from drying.

Cross link at 1, 200mJ for ~25 sec (choose auto cross linking on a Stratalinker 1800, Stratgene, CA)

Proceed directly to prehybridization.

If to be stored, allow membranes to air dry. Dry membranes can be stored at –20C. Before using dry membrane for hybridization, wet in 2 X SSC before proceeding to prehybridization. Representative RNA samples fixed to GeneScreen membrane are shown below. Samples are from four intestinal segments and two piglets



TRANSFECTION, PLASMID EXTRACTION, AND NORTHERN HYBRIDIZATION.

E2 Transfection

The aim of this process is to generate large amount of bacterial cells with plasmid containing the probe of interest. The process, although described here for a particular probe, can be modified and used for any DNA probe.

Plasmid DNA encoding rat $E2_{14k}$ cDNA was a generous gift from Dr Simon Wing, Polypeptide Research Laboratory, McGill University, Montreal Canada. The E2 sequence

(ca 480 b) is inserted at the SmaI site in the multicloning region in a pTZ18R (Pharmacia) plasmid backbone.

Competent cells used for the transfection can either be made in the laboratory (see below) or purchased from Stratgene as XLI-Blue MR supercompetent cells.

Preparation of competent cells

Needed: LB medium (see below), $CaCl_2$, glycerol, 50-mL centrifuge tubes

5 mL of XLI Blue cells are grown overnight at 37C
Grow 1mL of this per 100mL of LB (see below) medium for 3 h at 37C (see below)
Chill for 30 min and put into two 50mL centrifuge tubes. Centrifuge for 8 min at 5500 rpm (3460 x g) using Rotor JA21 (Beckman Centrifuge, model J2-21) 4°C.
Resuspend each tube in 12.5mL of ice cold 0.1M $MgCl_2$. Keep on ice for 10min
Spin for 8 min at 5500 rpm (JA21)
Resuspend in 25 mL of 0.1M $CaCl_2$ and keep on ice for 20 min
Centrifuge for 8 min at 5500 rpm
Resuspend pellet in 6 mL of freezing solution (8.6 mL of 0.1M $CaCl_2$ plus 1.4mL of glycerol)
Keep overnight on ice

Transfection

Materials: Ampicillin treated culture plates, 12-mL falcon tubes,

Solutions:
LB Medium (250 mL):

Bacto-tryptone (DIFCO, Cat # 0123-17-3)	2.5g
Bacto-yeast Extract(DIFCO Cat #0127-17-9)	1.25g
NaCl	2.5g

pH to 7 with 5N NaOH. Adjust the volume. Autoclave for 20 min and store sterile

Ampicillin Plates:

Make 7.5% agarose in LB medium (7.5 g in 100mL). Melt the agarose in an autoclave machine.

To coat with ampicillin and methicillin (antibiotics): Although ampicillin is the standard antibiotic used, methicillin is thought to help prevent the growth of unwanted satellite cells that might be resistant to ampicillin. Use 50 µg of ampicillin and 80 µg of methicillin per mL of LB medium.

Pour the LB-agarose mixture (with or without antibiotics) into petri dishes and allow to solidify. Store at 4°C.

1. Make sure ampicillin-treated plastic petri-dishes (for culturing) are put in 37°C incubator for at least 2 h before streaking the plate.
2. Pipette 1 µl of plasmid into 12 mL-sterile falcon tube (the type used in RNA isolation)
3. Take competent cell suspension (XL1 Blue *E. coli*) from –70°Cfreezer and allow to thaw for 15 minutes on ice. DO NOT take the cells out of the ice bucket. At the same time set water bath to 42°C.
4. Add 100 µl of cell suspension into the falcon tube that already contained the plasmid. Allow to sit on ice for 30 minutes. DO NOT take the tube out of the ice bucket. You can gently mix contents, but ALWAYS on ice.
5. Disrupt cell membrane by dipping the tube in the water bath (42°C) and hold it there for 45 seconds.
6. Take out and sit it on ice for 2 minutes.
7. Add 100 µl **antibiotics-free** Broth (e.g., LB medium) and place in the incubator (37°C) in a shaker for 45 minutes.

Streaking transformed cells on Agarose-Nutrient plates.

1. Sterilize a metal piece by dipping in ethanol and then passing it over flame. Allow to cool
2. Take the antibiotic-treated LB medium petri dish (see above) out of the incubator and pour the content of the falcon tube (step 8 above) into the petri dish.
3. With the aid of the streaking metal, spread the cells evenly in the dish.
4. Put the dish in the incubator overnight (about 16 h).

Plasmid Extraction

Materials: Plasmid Extraction Kit (PlasmidPure MINIPREP Kit, Sigma, St. Louis, MO)

1.5mL microcentrifuge tubes

TE Buffer: 10 mM Tris HCl
0.1 mM EDTA
pH should be 8.0

1. Inoculate a single bacterial colony from the Ampicillin plate (in the last section above) into 5.0 mL of LB medium in a culture tube and grow in the presence of Ampicillin (stock solution is 50µg / µL, add 15 µL of stock per 5 mL of LB medium). Incubate at 37°C in a shaker for about 16-18 h.
2. Next day collect as much as you can of the bacterial cells in a 1.5 mL microcentrifuge tube and centrifuge at 12,500 rpm (top speed in most microcentrifuges). Remove the supernatant, spin again and remove and discard the excess medium. (**N.B.** Since the volume of medium is 5 mL and microcentrifuge tube can only take 1.5 mL, first add ~ 1.5 mL of cells into the tube, centrifuge, remove supernatant, add the next 1.5 mL, centrifuge again, repeat the process so that all the pellet from 5 mL of cells are collected in one microcentrifuge tube. After the last centrifugation (i.e., all the pellet from 5 mL of cells has been collected), repeat the centrifugation, collect and discard the excess medium).

3. Use Plasmid Extraction Kit (A, B, C, and wash solutions as well as PlasmidPure spin filter and tubes are from the kit). Resuspend the pellet in 250 µL of Resuspension Solution A by either vortexing or pipetting up and down until no visible clumps of cells are observed. **The final yield of plasmid is affected by the efficiency with which the pellet is resuspended.** In re-suspending the pellet care must be taken not to break the DNA strands. This may contaminate the plasmid.
4. Before use, check Lysis solution B for precipitation. If necessary redissolve SDS by hand warming or incubation at 37C for a few minutes. Add 250 µl of Lysis Buffer B to the resuspended pellet and mix gently by inverting the capped tube 10-12 times. The lysed cell suspension should look clear. If it is cloudy, continue mixing until suspension clears.
5. Add 250 µl of Neutralization solution C to the lysed cells and mix by gently inverting the capped tube 10-12 times. The solution coagulates and has a visible, off white, floating precipitate.
6. Centrifuge at 12, 0000g for 5 min to form a while pellet along the side or at the bottom . If a gelatinous mass is present, lysis was incomplete and the plasmid yield will be low.
7. Place plasmid pure spin filter in a 2.0mL centrifuge tube and pour the cleared supernatant directly into the filter and centrifuge at 12000g for 1 minutes.
8. Remove the PlasmidPure spin insert from the microcentrifuge tube, discard the filtrate and replace the filter in the same tube. Add ml of 1X Wash Solution D and centrifuge at 12, 000g for 2 min.
9. Repeat wash procedure as in step 7 with a two minute centrifugation at 12000g to completely dry the filter of ethanol
10. Remove the Plasmid Pure spin filter, discard the microcentrifuge tube and place the filter in a fresh tube. Add 50 µl of deionized water. Incubate at room temperature for 1 minute. Centrifuge at 12000g for 1 minute.
11. The eluted DNA can be stored at 4C for immediate use or –20C for long term storage. For long term storage, elute in Elution Buffer (TE Buffer)

PROBE PREPARATION AND HYRBDIZATION

Materials:

Restriction Enzymes (see below), Molecular Weight Marker (Boehringer Mannheim Cat. #2382-50)

0.5 X TBE buffer, Molecular Biology Grade Agarose, Gene Clean Kit (Bio / Can Scientific, CA Cat # 1001-400), Orange G dye, Ethidium Bromide;

Random Primers DNA Labeling Kit (Life Technologies, Cat. #18187-013); Polyvinylpyrolidone (M Wt. 360, 000,Sigma Cat. # P-5288), Ficoll 400 (Sigma F-2367), Diethyl Pyrocarbonate (Sigma Cat. # D-5758), Sodium dodecyl sulphate (SDS, BDH Cat. # B30175); Bovine serum albumin (BSA, fraction V, Sigma Cat. # A-7906); Sephadex G 50 (fine, Sigma Cat. # G-50-80), ^{32}P dATP, Glass wool (Fisher Cat. # 11-390), Autoradiogram film (X-OMAR™-AR film, Kodak, Rochester, NY Cat. # 165 1454).

Solutions:

Orange G dye:

4.5 mL 30% Ficoll

1.0 mL Orange G (2%, Sigma Cat. #O3756)

0.65 mL 0.5 M EDTA

0.5 mL ddH_2O

TE Buffer pH 8.0

2.0 mL 1 M Tris-HCl pH 8.0

0.4 mL 0.5 M EDTA pH 8.0 (see below)

qsp 200 mL

autoclave 20 min and cool

adjust volume with sterile H_2O

EDTA 0.5 M pH 8.0

37.22 g EDTA disodium salt (MW 372.2)

adjust pH with NaOH pellets (allow to cool)

qsp 200 mL

autoclave 20 min and cool

adjust volume with sterile H_2O

Tris-Borate (TBE) Buffer

To make 1L of 5X: 54 g Tris base (Sigma Cat # T8524)

27.5 g boric acid

20 mL 0.5 M EDTA (pH 8.0)

Denhardt 50X

Ficoll 400 : 1%

Polyvinylpyrolidone 1% (mw 360,000)

BSA (fraction V): 1%

10 g ficoll

10 g PVP

10 g BSA

qsp 1 L (this is a lot)

do not autoclave

filter through .2 micron filter (e.g., Nalgene filter unit Cat # 1250020)

aliquot into 15 mL sterile Falcon tubes

store @ -20°C

Diethyl Pyrocarbonate (DEPC) H_2O

2 L deionized H_2O (use 2.5 L Winchester bottles)

in fume hood add:

2 mL DEPC (stored at 4°C)

cover and mix overnight in fume hood

autoclave

solution is good for ~ 1 month

10% SDS

10g SDS in 100 mL autoclave ddH_2O

Warm (autoclave) to 65°C

1 M Tris-Cl pH 7.5

Note: This buffer takes about half a day to make. Not all electrodes are good to make tris buffers.

24.23 g Tris base (MW 121.14)

qsp ~ 180 mL with DEPC treated H_2O

adjust to pH 7.5 with conc. HCl (~13 mL)

allow solution to come to room T before making the final adjustment: Tris buffers are very temperature dependent

qsp 200 mL with DEPC treated H_2O

autoclave 20 min and cool

adjust volume with DEPC treated H_2O

Sephadex G50 in TE Buffer

Soak sephadex in TE buffer (1 g / 9-11mL). Allow to sit overnight under vacuum. Add more buffer and store at 4°C

Prehybridization & Hybridization Solutions

30 mL per large hybridization tube (15 mL prehyb & 15 mL hyb)

	for	30 mL	60 mL
Denhardt 50X		6 mL	12 mL
5 M NaCl		6 mL	12 mL
1 M Tris-Cl, pH 7.5		1.5 mL	3 mL
5% Na-PPi		0.6 mL	1.2 mL
sterile H_2O		12.75 mL	25.5 mL
10% SDS (add last)		3 mL	6 mL

mix and place in hybridization oven to warm

at last minute add:

salmon sperm DNA (denatured)		150 µL	300 µL
	total	30 mL	60 mL

Procedure

Digestion

The probe can be prepared either by linearizing the DNA with BamH I and then using a riboprobe labeling kit, or the insert can be excised by Kpn I and BamH I and then labeled by random priming. The later method was used. The E2 insert is excised by

1) Kpn1 (GIBCOBRL, Life Technologies, Cat No:15232-010, Lot No: JDP405). The batch contained 2000 units; 10U/µl .

The cleavage sites for the enzyme are 5'-G GTAC↓C-3'
3'- C CATG↑G-5'

2) BamH I (GIBCOBRL, Life Technologies, Cat No: 15201-031, Lot # JHW412). The batch contained 10, 000 units; 10U/µl. Restriction sites

5'G↓GATC C-3'
3'C CTAG↑G-5'

Although optimum buffer media for the two enzymes are REACT 3 (BamH I) and REACT4 (KpnI), the best buffer for the two, is REACT 4. The two buffer solutions came with the enzymes. REACT 4 was used as described below

Cut the insert using the following protocol
10 µl of plasmid DNA, 5 µl of 10X buffer REACT 4, 2.5 µl KpnI, 2.5 µl BamH I, 30 µl of sterile water. Total volume is 50 µl. Place in 37C for ≥ 2h.

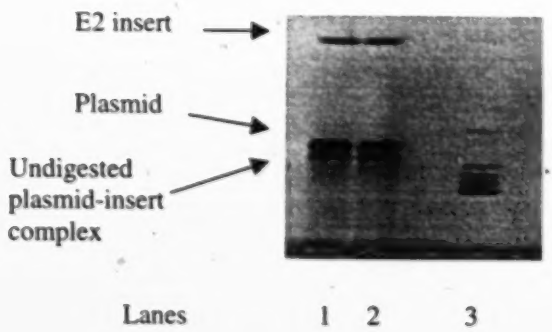
Afterwards, run the entire reaction mixture on a 1% agarose gel in 0.5XTBE buffer as follows:
In to a 150 mL conical flask, weigh 0.35g Agarose. Add 35 mL 0.5 X TBE buffer. Put in microwave oven to melt, care being taken to allow the solution to overflow. Cool under the tap to ~55C. Pour into a mini size electrophoresis unit with the comb and bridges in place. Allow the gel to solidify.

While waiting, add 20 µl orange G dye to the reaction mixture. Before using, warm the molecular weight marker at 67C for ~3 min. The marker is DNA molecular weight marker 11 from Boehringer Mannheim (Cat # 2382-50). It contains 8 fragments (0.12-23.1kbp: 125, 564, 2027, 2322, 4361, 6557, 9416, 23130bp). Orange G dye is added to the marker too.

After the gel has solidified, cover it with 0.5 X TBE buffer, remove the bridges and the comb, then load the sample (reaction mixture with dye) and the DNA marker. Depending on the size of the comb, one may be able to load the entire sample in 1, 2 or 3 lanes. The marker is loaded in another lane, leaving at least one lane between sample lane/s and the marker. Run for 1 h or till after the different molecular marker bands and reaction products have been separated.

Before viewing under UV light, stop the electrophoresis, remove the gel and stain with ethidium bromide: **use 25 µl /100ml ddH_2O from a stock of 10 mg/mL.** Place the gel in the staining solution with gentle shaking for 20 min. View it under UV light. If the bands have sufficiently separated, proceed as below. If not return the gel to the electrophoresis unit and let it run for more time.

Take a picture of the gel and print for the record. Representative sample of a typical digestion is shown below.



Lanes 1 and 2 are the reaction mixtures while lane 3 is a molecular weight marker. Although 3 bands are seen in the sample lanes, (representing the insert, the plasmid backbone (without the insert), and the plasmid-insert complex), if the digestion is complete, only two bands will be seen. These will be the E2 insert and the plasmid backbone.

Isolation of Insert

Carefully excise the band containing the insert (ca 500bp). Weigh the agarose band and proceed as follows, using the GENE CLEAN kit. All the solutions referred to in the steps below (NaI, glassmilk suspension, New wash) came with the kit

1. Add 4.5 volumes (weight of the gel) NaI stock solution and ½ volume of TBE modifier in a 1.5 mL microcentrifuge tube. Incubate for 5 min at 45-55C to dissolve the agarose. If the gel does not dissolve, invert several times and return to the incubator until the gel dissolves completely.
2. Prior to use, mix the glassmilk suspension several times before pipetting the suspension. Add 5 µl glassmilk suspension and incubate for 5 minutes on ice with periodical mixing to ensure the DNA binds to the silica in the glassmilk
3. Pellet glassmilk / DNA complex for 5 sec in a microcentrifuge. Remove the supernatant and set aside.
4. Before use, dilute NEW WASH, following the manufacturers' instructions as appropriate. Wash pellet 3 times with diluted NEW WASH, each time using 300-500 µl.
5. Remove every last bit of new wash after the last wash. Add **10 µl** of water and incubate for 3 min at 55C. Centrifuge as before and carefully remove the supernatant (which contains the DNA) leaving the glassmilk pellet behind.
6. The eluted DNA is ready to be used for Random Priming. Alternatively, one can check to be sure the DNA has not been lost during extraction. To do this, run 1 ml extract from step (5) combined with 9µl ddH2O and 5 µl dye on a minigel electrophoresis unit. Stain with ethidium bromide and view under UV light. If the

band is seen, the rest of the insert can be used. If not, the entire insert extraction could be flawed in which case a new extraction is needed.

Random Primer DNA labeling

1. Combine 5 µl DNA from step (5) above with 18 µl of ddH_20 in a microcentrifuge tube (1.5 mL size) and heat for 5 minutes in a boiling water bath. Then cool immediately on ice
2. Perform the following additions on ice:

   2 µl dCTP

   2 µl dGTP

   2 µl dTTP

   15 µl Random Primer Buffer Mixture. Spin down briefly.

   Before proceeding, put on double gloves and work behind plexiglass shield. The reaction tube should also be in an appropriate box designed for the purpose. ^{32}P is a medium energy β-emitter and is extremely penetrating. You can not be too careful when working with this isotope. Before and immediately after using the isotope, you should use Geiger counter to monitor yourself and your environment.

   Add 5 µl (~50µCi)^{32}P dATP

   Total volume at this point should be 49 µl. Mix briefly.

3. Add 1 µl Klenow fragment (THE ENZYME MIXTURE: **must always be kept on ice**). Mix gently but thoroughly, e.g., by rinsing the pipette tip a few times.
4. Incubate for ≥ 1h at 25 C.
5. Some minutes before stopping the reaction, take Stop Buffer out from the freezer and place on ice to thaw. Add 5 µl of this to the reaction mix.

The probe is now ready for hybridization. If working with nitrocellulose membranes, you can proceed without purifying the probe. Nylon membranes (such as

GeneScreen™) however often generate blots with lots of background. Therefore the probe may be purified to remove free nucleotides before proceeding to hybridization. A further advantage of purifying the probe is that, because up to 50% of the final radioactivity may be associated with the free nucleotides, removing these ensures that in subsequent processes (hybridization, washing of membranes etc), one is exposed to greatly reduced amount of radioactivity.

MAKE PURIFICATION COLUMN

1. Put a small amount of glass wool in a beaker. Cover with aluminum foil and bake overnight. Store sterile.
2. Put a very small amount of baked glass wool in 1 mL syringe bottom. With a plunger, stack the glass wool to not more than 0.5 cm from the needle end of the syringe.
3. Using 3 mL syringe and long wide bore needle slowly add slurry of Sephadex G50 (FINE) in TE 1X buffer. Fill to within 1 cm of the top. Stack very well to avoid bubbles
4. Cover top with parafilm to avoid drying
5. Make column holders with 1.5 mL microcentrifuge tubes. Cut their lids of. Place one of the tubes in a 15 mL falcon tube. The 1.5 mL tube serves as the holder for the 1 mL syringe. Put the spin column into the falcon tube so that its end is in the microcentrifuge tube.
6. Rinse the column 3-4 times with ~300-500 µl 1 X TE buffer by adding the buffer to the open end of the column. Cover with the falcon tuber lid and spin at about 5000rpm (3080xg) for 4 minutes. Discard the collection in the microcentrifuge tube after each washing. After the last washing, spin again to get rid of excess TE buffer. Cover the column with parafilm to prevent drying. The column is now ready to be used.

PURIFY PROBE

1. Place a new microcentrifuge tube in the falcon tube. Put the column in place.

2. Before purifying the probe, take 1 µl of the final reaction solution for counting (total radioactivity, **T**).
3. Load the entire random priming reaction mix into the column (**behind a plexiglass shield!!!**). Cover with falcon tube lid and spin at 2500 x g for 4-5min.
4. The probe collects in the microcentrifuge tube while free nucleotides get trapped in the sephadex beads . Safely discard the column.
5. Take 1 µl of the probe for counting (probe radioactivity, **P**) and transfer the rest in to a new screw-capped microcentrifuge tube. Cap in readiness for boiling. See below.

QUANTIFY PROBE

1. Count the 1 µl aliquots for total (T) and probe (P) radioactivity. Count for 1 min in water. Percentage of probe radioactivity is P*100/T. This value should be about 50%. Total probe cpm should be $>0.5X10^7$ per one hybridization tube to get good signals.

2. Boil probe for 5 min and place on ice until ready to add probe to hybridization solution.

PREHYBRIDIZATION

1. Rinse hybridization tubes with 0.1N NaOH. Rinse thoroughly with autoclaved ddH_2O.
2. Make 30 mL hybridization solution per large tube (see recipe); put in hybridization oven @ 65°C
3. Rinse membrane in SSC 2X (dilute SSC 20X)
4. Empty hybridization tubes and rinse with SSC 2X
5. Roll up membrane and put in tube; unroll; membrane must be flat
6. Add 0.150 mL SS DNA (denatured) (for 30 mL)
7. Add 15 mL (pre) hybridization solution to tube
8. Put tube in oven for ≥ 1 h @ 65°C

HYBRIDIZATION

1. During the prehybridization steps, finish making the probe;

Do not forget to boil the probe!! and put on ice for 10 min

2. Pour out prehybridization liquid

3. Carefully add probe to prehybridization solution, swirl and add to prehybridization tube

4. Return to hybridization oven and leave overnight (16 h) @ 65°C

WASH MEMBRANES

The next day wash membranes: 3 to 4 washes of 0.2X SSC and 0.1 % SDS @ 100 mL (large tube) per wash for 15 min @ 65°C

1. Make washing solution and warm to 65°C
2. Empty hybridization tube into container behind shielding
3. Add 100 mL warmed wash solution (large tube)
4. Wash 15 min @ 65°C
5. Empty (into container) and repeat wash 2 to 3 times. Monitor background with Geiger counter.
6. Remove membranes and put between sheets of plastic wrap
7. Seal membranes in plastic bag

MAKE AUTORADIOGRAMS

1. Tape membranes to a cassette with two intensifying screens
2. Go to dark room. Turn on red light and turn off normal light.
3. Tape a sheet of autoradiographic film (X-OMAR™-AR film, Kodak, Rochester, NY) on to membranes. Mark corner with felt pen to know the orientations of the membrane and film. Cover with intensifying screen.
4. Close cassette and cover with green garbage bag
5. Put cassette @ -80°C until ready to develop

DEVELOP AUTORADIOGRAMS

Make sure there is enough solution in the solution tank.

Turn developer on 30 min ahead of time. Also turn tap water on.

Process when the developer is ready. Process one or two blank films

Load films.

SCAN AUTORADIOGRAPHS

Use Biorad-Imaging Densitometer (GS-670) with Molecular Analyst software.

DEHYBRIDIZE MEMBRANES

Membranes may be dehybridized and rehybridized 3 or 4 times

1. Rinse small Pyrex dish with DEPC treated H_2O
2. Add 500 mL dehybridization solution

1% SDS	50 mL 10% SDS
0.1X SSC	2.5 mL 20X SSC
Sterile H_2O	450 mL

3. Cover with foil and boil
4. When boiling place membranes face-down in solution
5. Boil 5 min
6. Remove and place in plastic wrap.

Appendix 3

Preliminary Experiment and Data for Chapter 4

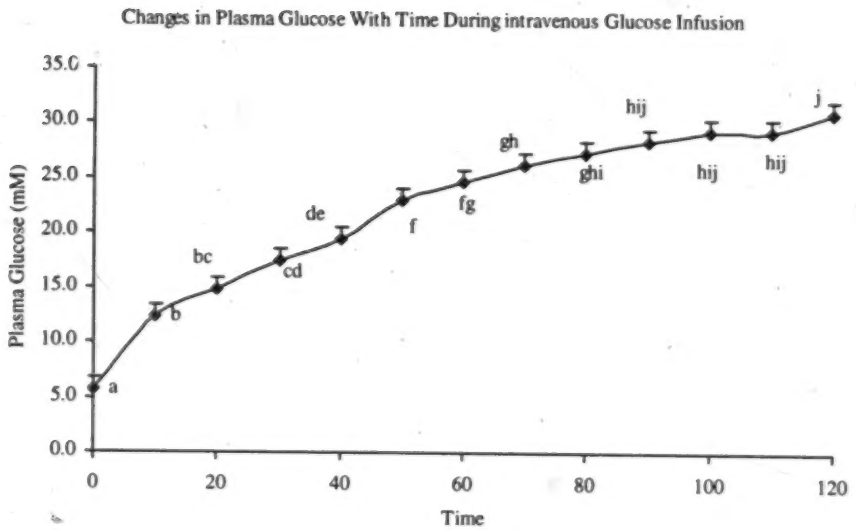


Fig. A-3. Three piglets were intravenously infused with glucose (20 mg / Kg body weight / h) for two hours. Blood samples were taken before the start of infusion and thereafter at ten-minute intervals. Plasma samples were analyzed for glucose concentration. Based on the result of this experiment, the length of infusion in experiment described in Chapter 4 was fixed to fall within the arrowed section of the figure.

TABLE A-3

Effects of Intravenous and Luminal Nutrients on the Expression of Proteolytic Genes[a]

Intravenous Treatments

Treatments	Glucose infusion	Saline Infusion	Pooled SE	P value
Proteolytic Genes				
Ubiquitin	34.59	28.41	3.2	0.26
14 kDa E2	12.87	10.14	0.9	0.11
20S Proteasome				
C8-subunit	10.25	5.23	1.7	0.13
C9-subunit	4.02	3.05	0.4	0.21
m-Calpain	10.34	12.82	1.0	0.15

Luminal Treatments

Luminal treatments	PBS	30mm Amino acid + 50mm glucose	30 mm Amino acid	30 mM Gln	Pooled SE
Proteolytic Genes					
Ubiquitin	37.80[b]	34.26[b]	27.13[c]	26.8[c]	2.3
14 kDa E2[d]	13.50[b]	13.28[b]	10.02[c]	9.24[c]	1.2
20S proteasome					
C8-subunit	6.45[b]	8.48[b]	8.20[b]	7.84[b]	1.4
C9-subunit	4.18[b]	3.97[b]	2.95[c]	3.04[c]	0.3
m-calpain	10.73[b]	12.22[b]	12.52[b]	10.84[b]	0.8

[a]See Chapter 4 for description of the experiment. [bc]Values within a row having the same superscripts are significantly different from one another ($P < 0.5$). [d]14 kDa ubiquitin conjugating enzyme.